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THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXVII, No. 4 APRIL 1, 1923

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

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The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

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WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

THE DISTRIBUTION OF RICKETTSIA IN THE TISSUES OF INSECTS AND ARACHNIDS.

By E. V. COWDRY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 25 TO 27.

(Received for publication, November 6, 1922.)

INTRODUCTION.

This preliminary survey of the distribution of *Rickettsia* was commenced in the hope of ascertaining what factors are responsible for the occurrence of such bodies and in order to glean further information bearing upon the claim that they constitute a new class of microorganisms distinct from bacteria.[1–4]

Technique.

In accordance with the advice of all authors who have studied *Rickettsia*, Giemsa's stain (as manufactured by Grübler) was used as the chief routine means

[1] da Rocha-Lima, H., *Berl. klin. Woch.*, 1916, liii, 567; Jungmann, P., Das wolhynische Fieber, Berlin, 1919; and other investigators.

[2] In a problem of this kind no progress could have been made without widespread cooperation in the collection of material. I wish to express my thanks to the following persons for their assistance: Dr. R. G. Ayres, Emergency Hospital, Honolulu, Hawaiian Islands; Dr. W. Reid Blair, New York Zoological Park, N. Y.; Mr. F. Bradshaw, Department of Agriculture, Regina, Saskatchewan, Canada; Dr. W. H. Cadbury, Canton, China; Dr. E. N. Cory, College Park, Md.; Mr. N. H. Cowdry, New York; Dr. S. R. Detwiler, Peking, China; Dr. W. P. Flint, State Natural History Survey Division, Urbana, Ill.; Dr. Laura Florence, The Rockefeller Institute, Princeton, N. J.; Dr. E. Pegram Flower, Baton Rouge, La.; Dr. E. H. Fullaway, Department of Agriculture, Honolulu, Hawaiian Islands; Dr. R. W. Harned, Agricultural College, Miss.; Dr. W. C. Hausheer, International Health Board, Dutch Guiana; Dr. C. W. Hobbs, Kansas State Agricultural College, Manhattan, Kan.; Dr. H. Hurlebaus, Southerland, Fla.; Dr. Edward C. Kirk, Philadelphia, Pa.; Dr. W. K. Lewis, Clemson Agricultural College, Columbia, S. C.; Dr. S. H. McNutt, Ohio State College, Ames, Ohio; Dr. A. L. Melander, Washington State College, Pullman, Wash.; Dr. Z. P. Metcalf, North Carolina State

of coloration. It was employed as directed by Wolbach.[5] The addition of traces of colophonium to the alcohol of differentiation was helpful in increasing the red coloration and it was found that the blue tint could be increased at will by leaving out the alkaline bath before staining and by dispensing with the addition of methyl alcohol to the diluted stain. After a little experimentation, sections were used in preference to smears, and since they were cut 4μ in thickness, differentiation was accomplished in less than 1 minute.

Zenker's fluid with and without acetic acid was employed as a fixative, but it was on the whole found to be less satisfactory than Regaud's fluid, which consists of four parts of 3 per cent potassium bichromate and one part of commercial formalin. This gave a clearer background with less artificial granulation. The high concentration of formalin, amounting to 20 per cent, was helpful in penetrating between the plates of chitin, and the bichromate served as a mordant for Giemsa's stain. It possessed the additional advantage of being a good fixative for mitochondria and other cellular components.

When it was impossible to obtain the insects alive they were shipped to New York in 10 per cent formalin and were mordanted over night in Regaud's fluid before being imbedded the next day. Larger forms, like ticks and bedbugs, were eviscerated and the body contents immersed in the fluid, while smaller

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College of Agriculture and Engineering, Raleigh, N. C.; Dr. M. Miyajima, Tokyo, Japan; Dr. V. A. Moore, New York State Veterinary College at Cornell University, Ithaca, N. Y.; Dr. Charles Murray, Ohio State College, Ames, Ohio; Mr. Newton, Regina, Saskatchewan, Canada; Dr. R. R. Parker, Hamilton, Mont.; Miss Elsie Preston, Agricultural College, Miss.; Dr. John L. Rice, International Health Board, Princes' Town, Trinidad; Dr. Hartwell Robbins, Washington, N. C.; Professor J. J. Sanders, Bureau of Plant Industry, Harrisburg, Pa.; Dr. Dwight L. Sisco, International Health Board, St. Johns, Antigua; Dr. Ernest W. Smillie, The Rockefeller Institute, Princeton, N. J.; Professor Otto Swezey, Sugar Planters' Association, Honolulu, Hawaiian Islands; Sir Arnold Theiler, Director of Veterinary Education and Research, Pretoria, Union of South Africa; Mr. Troy Thompson, Agricultural College, Miss.; Dr. W. B. Trimble, New York; Dr. Eugene Walker, Hamilton, Bermuda; and Dr. B. E. Washburn, International Health Board, Kingston, Jamaica.

[3] The courtesy of Professor S. B. Wolbach in giving me preparations showing the parasite of Rocky Mountain spotted fever and assistance from the following members of the Bureau of Entomology in the identification of specimens are gratefully acknowledged: Dr. L. O. Howard, Chief of the Bureau, Dr. A. L. Quaintance, Acting Chief during the summer of 1922, and Dr. J. M. Aldrich, Dr. F. C. Bishopp, Dr. Caudell, Dr. H. G. Dyar, Dr. H. E. Ewing, Dr. W. L. McAtee, Dr. W. W. Mann, Dr. S. A. Rohwer, Dr. E. A. Schwarz, and Dr. Clarence R. Shoemaker.

[4] All other determinations were made by Mr. N. H. Cowdry.

[5] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 185.

specimens, like fleas and some lice, were fixed *in toto*, with the exception of their legs, and cut into serial sections. Reduction of the time of dehydration to 2 or 3 hours made it possible to section their chitinous coverings without undue difficulty.

Other fixatives and stains[6] were used for special purposes and living tissues were examined uncolored under direct and oblique illumination, and with supravital stains (methylene blue, neutral red, Janus green, etc.) for control.

OBSERVATIONS.

The first difficulty was to obtain a preliminary definition of *Rickettsia* to serve as a working basis.

According to Arkwright, Atkin, and Bacot,[7] they are:

"(*a*) Bodies of minute size, usually 0.5μ in diameter or less, of round or diplococcal shape, though very minute bacillary and even thread-like forms occur.

"(*b*) Though resembling very small bacteria in general appearance, they stain much less readily than ordinary bacteria but can be coloured with Giemsa's stain; they are readily decolourised by Gram's method.

"(*c*) Absence of motility.

"(*d*) Resistance so far, with one exception,[8] to attempts made to cultivate them on artificial media *in vitro*.

"(*e*) Their occurrence in very large numbers in the gut and in some cases in other organs of blood-sucking insects."

Still more recently Wolbach, Todd, and Palfrey,[9] while admitting that it is impossible to arrive at a satisfactory definition, emphasize the specificity of

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[6] Fixatives: 95 per cent alcohol (A), ammoniated alcohol (Aa), acetic sublimate (As), Altmann's fluid (Al), Bensley's A.O.B. (B), Bouin's fluid (Bo), Carnoy's fluid (C), da Fano's cobalt-silver nitrate (D), 10 per cent formalin (F), Giemsa's sublimate (Gs), 2 per cent osmic acid (O), Regaud's mixture (R), Zenker without acetic (Z), and Zenker with acetic (Za). Stains: aniline-fuchsin-methyl green (A), Biondi-Heidenhain (B), Ehrlich's triacid (E), fuchsin and methylene blue (F), Giemsa (G), Gram (Gr), iron-hematoxylin (H), Löffler's methylene blue (L), Löffler's flagella stain (Lf), pyronine-methyl green (P), Wright (W), W. H. Smith's capsule stain (WHS) according to formulas given by Mallory and Wright (Mallory, F. B., and Wright, J. H., Pathological technique, Philadelphia and London, 7th edition, 1918) and Lee (Lee, A. B., The microtomist's vade-mecum. A handbook of the methods of microscopic anatomy, Philadelphia, 8th edition, 1921).

[7] Arkwright, J. A., Atkin, E. E., and Bacot, A., *Parasitology*, 1921, xiii, 27.

[8] *Rickettsia melophagi*.[15]

[9] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922, and others.

Rickettsia for certain insect hosts and the fact that: "In every instance where careful study has been made it has been found—with the exception of the *Rickettsia* of typhus—that the organisms pass down through successive generations in the eggs."

It is evident from a review of the literature that investigators have for the most part contented themselves with brief comparisons of newly recognized organisms with *Rickettsia prowazeki*, and other more or less familiar forms, without attempting to specify reasons why they should be included under this general heading as distinct from ordinary bacteria. The presence of red- and blue-staining substances during the multiplicative phase, and the formation of an hour-glass-shaped constriction, have been observed only in a few instances and cannot therefore be regarded as group characteristics. The frequently noted tendency to be colored less intensely than ordinary bacteria with Giemsa's stain is difficult to estimate quantitatively and varies within wide limits.

To lend precision to this survey, stress is placed upon the ability of the organisms to lead an intracellular existence, their location in the tissues, their host specificity, their Gram-negative properties, and their bacterium-like morphology. By taking this action large numbers of bacterium-like organisms which are present only in the intestinal lumen are automatically excluded and the problem is immensely, though arbitrarily, simplified.[10]

111 carefully selected species of arthropods were examined, comprising representatives of 3 classes, 18 orders, and 59 families. They include sucking forms living entirely on mammalian blood; biting insects which depend for their nutriment upon epidermal scales, hair, and feathers, and only occasionally take blood which has escaped from abrasions; insectivorous and vegetarian forms; and species of the most varied habitat.

[10] In this survey they were found in 43 out of 111 species. Of 23 forms which were blood-feeding they occurred in 9, or a little over 39 per cent, which does not substantiate Hindle's (Hindle, E., *Parasitology*, 1921, xiii, 157) claim that the alimentary canal of blood-sucking insects is remarkably free from bacteria. Many bacteria, normal to the digestive tract of Hemiptera, have been described by Forbes (Forbes, S. A., *Bull. Illinois State Lab. Natural Hist.*, 1892, iv, 1).

Within a single species individuals from widely separate localities were studied to ascertain whether the *Rickettsia* are cosmopolitan in nature and to determine the influence of different climatic conditions. For example, New York bedbugs were compared with others from the Hawaiian Islands, China, and Jamaica, and from Antigua, Trinidad, and Dutch Guiana. Many of the species were represented only by one specimen, while in others, which proved to be more interesting, the observations were extended to include as many as 95 individuals, bringing up the total number examined to 656.

In order that the survey may be of constructive value in further work along this line, brief summarized statements are given of the negative as well as the positive findings, because failure to mention forms devoid of *Rickettsia* would leave the reader in doubt regarding the scope of the work and, in consequence, of its bearing upon the actual distribution of *Rickettsia*. In view of our ignorance concerning the causes underlying parasitism with *Rickettsia* these records are made to cover the locality, the season of the year, the technique, the sex, and any factors likely to influence the condition of the host, such as the presence of other bacteria and of protozoa. A large number of the specimens were collected in Woods Hole during the month of July;[11] fixed in formalin, followed by Regaud's fluid; and colored with Giemsa's stain,—which is the history of those about which no information is given in the summaries.

Acarus sp.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One female was negative.

Acrosoma spinea.—Two males and one female were negative.

Adalia bipunctata.—One female contained bacteria within intestinal lumen and body cavity.

Amblyomma americana (*Figs. 1 to 4*) (*A*).—Columbia, S. C., April, 1922; Dr. W. K. Lewis. Fixation, R. Staining, G, Gr, F, and L. Two females contained in one specimen: (1) Uniform straight rods, 0.2 to 0.3 by 1 to 1.5μ, with rounded ends, occasionally in diplo formation and often grouped in clumps with long axes sometimes parallel, stained deep purple with G, light blue with L, Gram-positive and not acid-fast, surrounded by very slight halos, intracellular in all tissues and without destructive action upon the cells. (2) Pleomorphic rods, 0.6 to 1 by 2 to 4μ, majority 1 by 3μ, with rounded ends, often curved and with marked tendency to be clumped, stained bluish purple with G and light blue

[11] These were collected by Mr. N. H. Cowdry.

with L, Gram-negative and not acid-fast, outlines often faint and surrounded by pronounced halos, abundant within egg cells, and apparently without destructive action.

(*B*).—Washington, N. C., April 29, 1922; Dr. Hartwell Robbins. Fixation, R. Staining, G. Five females showed Type 2 but it was less abundant.

(*C*).—Baton Rouge, La., May 29, 1922; Dr. E. Pegram Flower. Fixation, A, Al, As, B, Bo, C, Gs, and R. Staining, A, G, and H. In ten the eggs were noticeably less developed than in A. Only one female fixed in R and stained with G showed definite organisms like Types 1 and 2 in A. Staining with H revealed an abundance of very tiny mitochondria in the egg side by side with the organisms which became decolorized by treatment with iron alum much more readily than did the mitochondria (see Figs. 2 and 4).

Amblyomma hebræum.—Pretoria, Union of South Africa, June, 1922; Sir Arnold Theiler. Fixation, F and R. Staining, G and Gr. Thirteen males and seven females contained: (1) Uniform rods, 0.25 by 1μ, with rounded ends, purple with G, and Gram-positive within the cells of the salivary glands of two specimens. Some of the cells were swollen, granular, vacuolated, and unusually strongly stained with the red component of the dye. (2) Pleomorphic rods, 0.5 to 1 by 1.5 to 4μ, majority 1 by 3μ, slightly curved but unbranched, stained faintly with G, Gram-negative. Outlines faint and surrounded by marked halos, often clumped within egg cells, between egg cells, and within the cells of the Malpighian tubules.

Amblyomma maculatum.—Van Cleave, Miss., August, 1922; Mr. Troy Thompson (kindness of Dr. R. W. Harned). Fixation, F and R. Staining, G and Gr. Six females contained pleomorphic rods, 0.15 to 0.3 by 0.5 to 2μ, majority 0.2 by 1μ, with rounded ends and often curved, purple with G, and Gram-positive, in the eggs of four specimens, one of which contained them also within the cells and lumen of the Malpighian tubules, also intracellularly and extracellularly in central nervous system and intestine.

Amblyomma tuberculatum.—Perkinston, Miss., August, 1922; Miss Elsie Preston (kindness of Dr. R. W. Harned). Fixation, F and R. Staining, G and Gr. One female was negative.

Anisodactylus agricola.—Two females contained many bacteria within intestinal lumen.

Anystis agilis.—One female was negative.

Aphrophora saratogensis.—Two were negative.

Apis mellifica.—One contained almost a pure culture of bacteria within intestinal lumen.

Argas mineatus.—Southerland, Fla., June, 1922; Dr. H. Hurlebaus. Fixation, Z and R. Staining, G. Two were negative.

Arge erythrosoma.—One female was negative.

Arge sanguinea.—One female was negative.

Atomus sp. (*Fig. 5*).—Serial sections of eight females and seven of undetermined sex contained in one uniform diplococci about 0.4 by 1μ, dark red with G, and Gram-negative within large green pigmented hypoblastic cells.

Bombus vagans.—One contained many bacteria and plant spores within intestinal lumen.

Boophilus decoloratus.—Pretoria, Union of South Africa, June, 1922; Sir Arnold Theiler. Fixation, F and R. Staining, G, Gr, and WHS. Ten females contained in all but one instance, which was doubtful, highly pleomorphic filaments, 0.6 to 1 by 1 to 7.5μ, majority 0.6 by 5μ, wavy, branched (?), non-septate, light blue with G, Gram-negative, outlines relatively faint, surrounded by marked halos which were not differentially colored by WHS, clumped within egg cells, and in one specimen within cells of Malpighian tubules in addition.

Brachyrhinus ovatus.—One was negative.

Camponotus sp.—One showed many intestinal cells loaded with bacteroids very closely resembling those seen and recorded in *Periplaneta americana* and in *Hæmatopinus suis*, but more abundant.

Casinaria infesta (*Fig. 7*).—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G and Gr. One contained filaments 0.3 to 1 by 0.5 to 4μ, majority 0.3 by 2μ, with rounded ends, the longer ones curved, dark blue with G, Gram-negative, surrounded by slight halos, singly, rarely in clumps of ten or more within intestinal epithelial cells and body cavity.

Chlorion ichneumoneum.—One was negative.

Chrysopa oculata (*Fig. 6*).—Two, one of which contained rods, 0.2 by 0.5 to 1.2μ, majority 0.2 by 1μ, with rounded ends, purple with G, and Gram-negative within pigmented fat cells.

Chrysophanus hypophlæus.—One female was negative.

Cimex lectularius (*A*).—New York, December 1 and 8, 1921, and January 28, 1922. Fixation, Aa, Al, As, B, Bo, C, D, O, R, Z, and Za. Staining, B, E, G, L, and P. Serial sections of five, sections of five, and smears of five others all showed bodies conforming with the descriptions of Arkwright, Atkin, and Bacot. In addition, they were also discovered free in the lumen of the Malpighian tubules, as was predicted, but not observed, by them. That the bodies were independent of mitochondria and other cell constituents was established.

(*B*).—New York, July, 1922. Fixation, R. Staining, G. One was negative.

(*C*).—Honolulu, Hawaiian Islands, April 24, 1922; Dr. R. G. Ayres. Fixation, F and R. Staining, G. One showed, in addition to the above mentioned bodies, rod-like and oval bodies about 0.5 by 2 and 0.7 by 1.5μ respectively, stained dark blue, and exhibiting two spherical uncolored areas—a larger one (0.3μ) at one end and a smaller (0.2μ) at the other—widely distributed throughout the tissues. They were particularly abundant within fat cells and in the lining epithelial cells of the intestine. There was no cellular change other than great distention. Uniform rods, 0.3 by 1μ, in diplo formation, and stained dark red, were occasionally seen within the epithelial cells of the Malpighian tubules.

(*D*).—St. Johns, Antigua, May 4, 1922; Dr. Dwight L. Sisco. Fixation, F and R. Staining, G. Eight females and one male only showed bacteria within the body cavity of one female.

(*E*).—Trinidad, June, 1922; Dr. John L. Rice. Fixation, F and R. Staining, G. Twenty-five, of which eleven were male, four female, and ten undetermined, were negative.

(*F*).—Kingston, Jamaica, June, 1922; Dr. B. E. Washburn. Fixation, F and R. Staining, G. Eight males and eight females, of which five males and one female contained bacteria within the alimentary tract. Only one male contained a small clump of cocci (about 0.3μ) within one of the intestinal epithelial cells and closely resembling the *Rickettsia* frequently found in a similar location in New York bedbugs.

(*G*).—Peking, China, May, 1922; Dr. S. R. Detwiler. Fixation, F and R. Staining, G. Twenty-two contained in three cases typical organisms as in New York bedbugs; one specimen contained very large masses of bacteria within the body cavity.

(*H*).—Dutch Guiana, June, 1922; Dr. W. C. Hausheer. Fixation, F and R. Staining, G. One male and five females contained in one typical organism as in New York bedbugs.

Cincindela punctulata.—One female contained bacteria within intestinal lumen.

Colcodiscus lucipatens.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained a variety of bacteria within intestinal lumen.

Cryptocephalus sp.—One female was negative.

Cryptomorpha desjardinsii.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One was negative.

Ctenocephalus canis (*Fig. 8*).—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G and Gr. Serial sections of three males and one female showed in one male rods 0.2 to 0.3 by 0.4 to 1.5μ, majority 0.3 by 1μ with rounded ends, stained red with G, and Gram-negative, widely distributed intracellularly as follows: hypoblast, salivary glands, muscle, fat cells, testis, intestinal epithelium, Malpighian tubules and their lumina, etc.

Culex fatigans.—Honolulu, Hawaiian Islands, March and April, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. Serial sections of twelve showed in three protozoon-like bodies widely distributed through the tissues, in one spore-like bodies in the intestinal lumen, and in another numerous bacilli within the intestinal lumen.

Dermacentor albipictus.—Regina, Saskatchewan, Canada, December, 1921, and March, 1922; Mr. F. Bradshaw. Fixation, R. Staining, G. Two were negative.

Dermacentor variabilis (*Figs. 9 and 10*) (*A*).—Urbana, Ill., July, 1922; Dr. W. P. Flint. Fixation, F and R. Staining, G and Gr. Five females showed: (1) filaments 0.15 to 0.3 by 0.5 to 2μ, majority 0.3 by 2μ, with rounded ends, sometimes curved, red with G, and Gram-negative in the eggs and Malpighian tubule cells of one specimen and in the eggs of another; and (2) large masses of diplobacilli within rectal sac of one specimen.

(*B*).—College Park, Md., July, 1922; Dr. E. N. Cory. Fixation, F and R. Staining, G. Eight females all showed similar Gram-negative rods and filaments.

Dermacentor venustus (*A*).—Hamilton, Mont., May 11, 1922; Dr. R. R. Parker. Fixation, R. Staining, G and Gr. Six females, three males, and one of undetermined sex contained in seven specimens pleomorphic rods, 0.15 to 0.8 by 0.3 to 1.5μ, majority 0.25 by 1.2μ, with rounded ends, the longer ones often curved, sometimes in diplo formation, dark blue with G, and Gram-negative within intestinal epithelial cells, egg cells, muscle cells, etc.

(*B*).—Wawawai, Wash., about June 1, 1922; Dr. A. L. Melander. Fixation, R. Staining, G. Two, one of which contained bodies resembling the above mentioned widely distributed in the brain, salivary glands, eggs, muscle, intestinal lumen, etc.

Deromyia sp.—One was negative.

Dissosteria carolina.—One was negative.

Dolomedes urinator.—One was negative.

Dysdera interrita.—One was negative.

Echthromorpha maculipennis.—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One female contained bacteria within intestinal lumen.

Epeira trivittata.—One female was negative.

Erystalis æneus.—One female was negative.

Erystalis arbustorum.—One was negative.

Erystalis punctatus.—Honolulu, Hawaiian Islands, April, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One female contained many bacteria within intestinal lumen.

Erystalis transversus.—One contained many bacteria within intestinal lumen.

Erythemis simplicicollis.—One female contained streptococci scattered in clumps throughout the tissues.

Eumenes fraternus.—One male was negative.

Euschistus euschistoides.—One contained apparently a pure culture of bacteria within the intestinal lumen.

Gonocephalum seriatum.—Honolulu, Hawaiian Islands, April, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained bacteria within intestinal lumen.

Gryllotalpa africana.—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. Two contained bacteria of diverse morphology within intestinal lumen.

Gypona sp.—One male was negative.

Gyropus gracilis.—New York, April 13, 1922. Fixation, R. Staining, G. Serial sections of twenty-five were negative.

Hæmaphysalis leporis palustris.—Raleigh, N. C., April 3, 1922; Dr. Z. P. Metcalf. Fixation, F and R. Staining, G. Nineteen were negative.

Hæmatobia serrata.—Honolulu, Hawaiian Islands, February and March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. Serial sections of seven and one other were negative.

Hæmatopinus piliferus.—New York, November 22, 1921. Fixation, R, Z, and Za. Staining, G. Four were negative.

Hæmatopinus suis (*A*).—Princeton, N. J., February 8 and 9, 1922; Dr. Laura Florence and Dr. Ernest W. Smillie. Fixation, Aa, As, Bo, C, R, Z, and Za. Staining, G, Gr, and L. Serial sections of seventeen, and smears of eight others, all contained bacteroid-like organisms in intestinal epithelial cells very similar to those observed in *Periplaneta americana*. One contained clumps of cocci (0.6μ) in the lumen of the alimentary tract and another exhibited an infection of a Malpighian tubule with cocci (0.2μ) which invaded the cells and caused considerable destruction (vacuolation, etc.).

(*B*).—Manhattan, Kansas, March 3, 1922; Dr. C. W. Hobbs. Fixation, F and R. Staining, G. Serial sections of six from cases of hog-cholera were negative, apart from the bacteroids.

(*C*).—Ithaca, N. Y., May 16, 1922; Dr. V. A. Moore. Fixation, R. Staining, G and H. Serial sections of four from cases of hog-cholera were negative, except for bacteroids.

D.—Ames, Ohio, September, 1922; Dr. S. H. McNutt and Dr. Charles Murray. Fixation, F, R, and Z. Staining, G. Four males and thirty-five females contained, in two, masses of cocci, similar to A, within intestinal lumen and bacilli in six other cases.

Ilburnia ipomœicola.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One female contained, in the fatty tissues, bodies about 3 by 7.5μ, of oval shape, exhibiting considerable internal organization and suggestive of protozoa.

Isomera sericea.—One female was negative.

Isometrus maculatus.—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One was negative.

Lachnosterna sp.—One contained many bacteria within intestinal lumen.

Lebia sp.—One female was negative.

Leistotropus cingulatus.—One female was negative.

Lepisma saccharina (*Fig. 11*).—One male contained rods 0.2 by 0.2 to 0.7μ, majority 0.2 by 0.6μ, straight with rounded ends, red with G, and Gram-negative within intestinal epithelial cells and fat cells, and between the fibers in the nervous ganglia.

Lestes unguiculatus.—One showed tissues impregnated with bacteria but none clearly intracellular.

Lucoppia curviseta (*Fig. 12*).—Serial sections of five females and two males all contained fungi within intestinal lumen; one was heavily infected with protozoon-like bodies and one contained uniform rods 0.7 by 1μ in diplo formation, purple with G, and Gram-negative, within the large intestinal epithelial cells.

Lycosa pratensis.—One was negative.

Macrodactylus subspinosus.—One contained many bacteria within intestinal lumen and body cavity.

Malachis sp.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One female contained a few bacteria within the intestinal lumen.

Malthodes parvulus.—One female was negative.

Margaropus annulatus (*Figs. 13 to 15*) (*A*).—Columbia, S. C., April 14, 1922; Dr. W. K. Lewis. Fixation, R. Staining, G and Gr. Eight females contained: (1) in all cases, rods, 0.4 to 0.5 by 0.5 to 3.5μ, majority 0.5 by 2μ, with rounded ends, sometimes curved, purple with G, Gram-negative, often surrounded by halos, in clumps within egg cells; (2) in one specimen, spherules 0.5μ, and rods about 0.5 by 1μ, purple with G, and Gram-positive, found occasionally free in body cavity; and (3) in another, rods, 0.3 by 0.5 to 2μ, purple with G, Gram-negative, in the tissues between the cells.

(*B*).—Washington, N. C., April 29, 1922; Dr. H. Robbins. Fixation, R. Staining, G. Ten females contained bodies like Type 1 above mentioned in the egg cells, except that in some cases they attained a length of 5μ. When thus elongated, they tended to be arranged in clumps with long axes parallel. The Malpighian tubules and rectal sac in one were heavily infected apparently with the same organism. The lining cells became so distended that they ruptured and discharged organisms into the lumen.

(*C*).—Baton Rouge, La., May 2, 1922; Dr. E. Pegram Flower. Fixation, R. Staining, G. Two females were examined. The eggs contained bodies like the Type 1 organism but the Malpighian tubules were not infected.

Margaropus annulatus australis (*A*).—Jamaica, June, 1922; Dr. B. E. Washburn. Fixation, F and R. Staining, G and Gr. Four males and two of undetermined sex contained in four slender rods 0.6 to 1 by 3 to 8μ, majority 1 by 3μ with rounded ends, often curved, light blue with G, Gram-negative, surrounded by marked halos, in cells of Malpighian tubules and in two specimens disposed in clumps in the eggs.

(*B*).—Trinidad, July, 1922; Dr. John L. Rice. Fixation, F and R. Staining, G and Gr. Three females contained organisms like the above mentioned in eggs and cells of Malpighian tubules.

Megalotomus quinquespinosus.—One contained masses of streptococci within intestinal lumen.

Menopon pallidum.—New York, November 22, 1921, and January 12, 1922. Fixation, O, R, Z, and Za. Staining, G and Gr. Serial sections of four and sections of one other contained: (1) in the intestinal lumina of four specimens, diplococci 0.3 by 0.7μ, dark blue with G, and Gram-positive; and (2) within the intestinal lumina of the same specimens, smaller cocci, about 0.2μ, stained less intensely with G, and Gram-negative, associated with considerable destruction of the lining epithelial cells, but with no blood or blood pigment in the lumen.

Misumena vatia.—One contained an abundance of protozoon-like bodies.

Monobia quadridens.—One contained many filamentous bacteria within intestinal lumen.

Monomorium pharaonis.—New York, January 10, 1922. Fixation, R. Staining, G. Serial sections of three were negative.

Musca domestica.—New York, January 7, 1922. Fixation, R. Staining, G and Gr. One contained bacilli within intestinal lumen.

Mutilla sp.—One was negative.

Œchila grisea.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained: (1) oval bodies about 1.4 by 4.5μ, staining purple and showing marked internal organization, distributed in clumps in body cavity and in tissues surrounding the alimentary tube; and (2) cocci, diplococci, and bacilli in large numbers within the intestinal lumen.

Ornithodoros turicata (*Fig. 16*).—Arizona, March, 1922; Dr. W. Reid Blair. Fixation, R. Staining, G and Gr. One female contained pleomorphic rods, 0.5 to 1 by 0.5 to 2.5μ, majority 0.7 by 2μ, rounded ends, often in diplo formation, purple with G, Gram-negative, sometimes surrounded by halos in eggs and cells of Malpighian tubules.

Pallodes pallidus.—One female was negative.

Pantala flavescens.—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G and Gr. One contained a variety of bacteria, both Gram-positive and Gram-negative, within intestinal lumen and clumps of protozoon-like organisms widely distributed throughout the tissues both in and between the cells.

Pediculus humanus (*A*).—New York, January 7, 1922; Dr. W. B. Trimble. Fixation, R, Z, and Za. Staining, G. Serial sections of three and smears of six others were negative.

(*B*).—Serial sections of another taken in the month of June were negative.

(*C*).—St. Johns, Antigua, May 4, 1922; Dr. Dwight L. Sisco. Fixation, F and R. Staining, G. Serial sections of eight females showed in two cocci, 0.5 to 1μ, and bacilli, 0.5 by 2μ, purple with G in the lumen of the pharynx, but not extending into the stomach or intestines. Sections of several nits were negative.

(*D*).—Princes' Town, Trinidad, May, 1922; Dr. John L. Rice. Fixation, F and R. Staining, G. Serial sections of seven males and eighteen females contained in four females rods 0.2 by 0.6μ with faint outlines, sometimes in diplo formation, pink with G, and Gram-negative, strongly suggestive of *Rickettsia pediculi*, within intestinal lumen.[12]

(*E*).—Kingston, Jamaica; Dr. B. E. Washburn. Fixation, F and R. Staining, G. Serial sections of fifteen males and ten females showed only in one male a few extracellular cocci in the pharynx.

(*F*).—Peking, China, May, 1922; Dr. S. R. Detwiler. Fixation, F and R. Staining, G. Serial sections of nine males, sixteen females, and two of undetermined sex were negative.

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[12] No direct contact with Europe could be traced but a regiment from the island took part in the war.

(*G*).—Canton, China, December 28, 1922; Dr. W. H. Cadbury. Fixation, F. Staining, G. Eleven males and seven females were negative.

(*H*).—Hamilton, Bermuda, November 27, 1922; Dr. Eugene Walker. Fixation, F. Staining, G. Two males and eight females were negative.

Pelecinus polyturator.—One female was negative.

Pelidnota punctata.—One female contained large masses of diplobacilli within the segments of the alimentary tube.

Periplaneta americana (*A*).—New York, January 4 and 10, 1922. Fixation, Z and R. Staining, G. Two showed the usual bacteroids in the fat cells fully described by Glaser[13] and Hertig.[14]

(*B*).—Honolulu, Hawaiian Islands, April, 1922; Mr. N. H. Cowdry. One showed similar bacteroids.

Phalangium dorsatum.—Two in one case showed bacteria within intestinal lumen.

Photinus sp.—One was negative.

Pieris rapæ.—Honolulu, Hawaiian Islands, March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G and Gr. One showed Gram-positive sporulating bacilli within intestinal lumen and body cavity.

Polistes pallipes.—Of two females, one was negative and the other contained many bacteria within intestinal lumen.

Porthenia dispar.—One caterpillar was negative.

Promachus sp.—One was negative.

Psocus sp.—One was negative.

Pulex irritans (*Fig. 17*).—Trinidad, May, 1922; Dr. John L. Rice. Fixation, F and R. Staining, G and Gr. Serial sections of seven males and eighteen females, of which only one female contained: (1) rods, 0.2 by 0.5μ, with rounded ends, faintly outlined, purple with G, Gram-negative, in clumps within the intestinal lumen, but not observed within the epithelial cells; and (2) filaments 0.3 to 0.6 by 0.6 to 4μ, majority 0.3 by 3μ, with rounded ends, often curved, prominent halos, same staining reaction, within the body cavity and in the smaller fat cells; probably similar to Type 1.

Reduviolus sp.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained delicate rods about 0.2 by 0.8μ, purple with G, in clumps within the body cavity and in a layer of cells which invests the epithelial lining of the intestine. The lumen of the intestine contains the same bacilli and a fungus.

Rhipicephalus evertsi.—Pretoria, Union of South Africa, June, 1922; Sir Arnold Theiler. Fixation, F and R. Staining, G and Gr. Eighteen females and two males contained: (1) uniform rods, 0.5 by 1 to 2μ, with rounded ends, purple with G, and Gram-negative, in large numbers within intestinal lumen and occasionally penetrating lining cells in seven; and (2) pleomorphic filaments 0.6 to 1 by 2 to 6μ,

[13] Glaser, R. W., *Biol. Bull.*, 1920, xxxix, 133.

[14] Hertig, M., *Biol. Bull.*, 1921, xli, 181.

majority 1 by 4μ, with rounded ends, often curved, colored less intensely with G, and Gram-negative, within egg cells of seventeen and cells of Malpighian tubules of six.

Rhipicephalus sanguineus (*Figs. 22 and 23*) (*A*).—Honolulu, Hawaiian Islands, February and March, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, F, G, Gr, L, and P. Fourteen specimens contained pleomorphic rods 0.3 to 0.5 by 0.5 to 3μ, majority 0.3 by 2μ, with rounded ends, often curved, purple with G, lightly colored with P and L, Gram-negative, not acid-fast, within the cells of the Malpighian tubules in thirteen and in one in egg cells. The former were greatly enlarged and often ruptured with the discharge of organisms into the lumen.

(*B*).—Dutch Guiana, July, 1922; Dr. W. C. Hausheer. Six were negative, except for one which was doubtful.

Sallicus scenicus (*Figs. 18 to 21*).—One female contained filaments 0.2 to 0.3 by 1 to 5μ, majority 0.2 by 4μ, with curved, rounded ends, stained lightly with G, and Gram-negative, in the nervous system and within fat cells and egg cells. In the latter location they occurred with great constancy, one clump to each cell. Each clump was very compact, about the size of the nucleus, *i.e.* 35μ in diameter, and was surrounded by a dense capsule averaging 7.5μ in thickness. Six additional specimens taken 3 weeks later showed similar cysts in the egg cells of three females.

Satyrus alope.—One female contained many lightly staining bacteria within intestinal lumen.

Scolopendra repens.—Honolulu, Hawaiian Islands, February, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G and Gr. One contained diplococci and bacilli within intestinal lumen.

Scutigera forceps.—New York, January 12, 1922. Fixation, R. Staining, G. One was negative.

Sminthurus sp.—Two contained fungi and a variety of bacteria within intestinal lumen.

Spilopsyllus simplex.—New York, January 30, 1922. Fixation, R and Z. Staining, G. Serial sections of two and smears of one other were negative.

Sympetrum semicinctum.—Two were negative.

Tapinoma sessile.—One male was negative.

Telephorus lineola.—One female contained fungi within intestinal lumen.

Termes sp.—Honolulu, Hawaiian Islands, April 24, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained a few bacteria within the intestinal lumen.

Theriodion tepidariorium.—New York, January 12 and 18, 1922. Fixation, Z and Za. Staining, G. Two were negative.

Trombicula akamushi.—Japan, Dr. M. Miyajima. Fixation, A. Staining, G. One adult was negative.

Tychius picirostris.—One contained great masses of bacteria within intestinal lumen and body cavity.

Urographis fasciata.—One contained many bacteria within intestinal lumen.
Vespa maculata.—One contained many bacteria within intestinal lumen.
Xylocarpa varipunctata.—Honolulu, Hawaiian Islands, April, 1922; Mr. N. H. Cowdry. Fixation, F and R. Staining, G. One contained bacteria within the intestinal lumen.

The survey shows that intracellular, Gram-negative, bacterium-like *Rickettsia* are not of particularly rare occurrence, since they were found in 19 out of 111 species examined; that is to say, in about 17 per cent. This may be contrasted with the presence of bacteria within the intestinal lumen in 39 per cent. Of these nineteen types, two, occurring in *Dermacentor venustus* and *Cimex lectularius*, are described in the literature, while the remaining seventeen, occurring in *Amblyomma americana* (Figs. 1 to 4), *Amblyomma hebræum*, *Atomus sp.* (Fig. 5), *Boophilus decoloratus*, *Casinaria infesta* (Fig. 7), *Chrysopa oculata* (Fig. 6), *Ctenocephalus canis* (Fig. 8), *Dermacentor variabilis* (Figs. 9 and 10), *Lepisma saccharina* (Fig. 11), *Lucoppia curviseta* (Fig. 12), *Margaropus annulatus* (Figs. 13 to 15), *Margaropus annulatus australis*, *Ornithodoros turicata* (Fig. 16), *Pulex irritans* (Fig. 17), *Rhipicephalus sanguineus* (Figs. 22 and 23), *Rhipicephalus evertsi*, and *Salticus scenicus* (Figs. 18 to 21), have not hitherto been reported. If the arbitrary definition already given be adhered to, at least two other *Rickettsia* which are described in the literature in *Pediculus humanus*[1] and one in *Melophagus ovinus*[15] can be added, making a total of twenty-two. The intracellular organisms described in *Cimex hirundinus*,[7] *Ctenocephalus felis*,[16] and *Ctenopsylla musculi*[16] are probably Gram-negative, but until definite information is forthcoming on this point their status remains uncertain. There is every reason to believe that many other *Rickettsia* will be found though not in so high a percentage unless the species examined are recruited in like proportion from the several families, because in the present survey a large number of blood-feeding forms are included in which they are particularly abundant. Only the barest outline of their actual distribution may be arrived at from so brief a study in consideration of the hundreds of thousands of species concerning which nothing is known.

[15] Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53.
[16] Sikora, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1918, xxii, 442.

In the following systematic list all the Arachnida, Myriapoda, and Hexapoda examined are noted and those found to contain *Rickettsia* are given in capitals as are also the above mentioned species described in the literature. If a larger number of individuals in each species had been examined it is quite likely that *Rickettsia* would have been found in species which are here reported negative. Similarly, if individuals had been taken at regular intervals throughout the year, in more localities, and in a wider range of physiological conditions, or had been examined more closely in more perfect sections, *Rickettsia* might well prove to be of more general occurrence. In other words, the actual distribution in the 111 species examined is probably greater than is indicated.

Arachnida.

Scorpionida.		*Isometrus maculatus.*
Phalangidea.		*Phalangium sp.*
Araneida.		*Dysdera interrita.*
	Theridiidæ.	*Theriodion tepidariorium.*
	Thomisidæ.	*Misumena vatia.*
	Epeiridæ.	*Acrosoma spinea; Epeira trivittata.*
	Lycosidæ.	*Dolomedes urinator; Lycosa pratensis.*
	Attidæ.	SALTICUS SCENICUS.
Acarina.	Trombidiidæ.	*Acarus sp.; Anystis agilis;* ATOMUS SP.; LUCOPPIA CURVISETA; *Trombicula akamushi.*
	Argasidæ.	*Argas mineatus;* ORNITHODOROS TURICATA.
	Ixodidæ.	AMBLYOMMA AMERICANA; AMBLYOMMA HEBRÆUM; *Amblyomma maculatum; Amblyomma tuberculatum;* BOOPHILUS DECOLORATUS; *Dermacentor albipictus;* DERMACENTOR VENUSTUS (Wolbach[5]); DERMACENTOR VARIABILIS; *Hæmaphysalis leporis palustris;* MARGAROPUS ANNULATUS; MARGAROPUS ANNULATUS AUSTRALIS; RHIPICEPHALUS EVERTSI; RHIPICEPHALUS SANGUINEUS.

Myriapoda.

Chilopoda.	*Scolopendra repens; Scutigera forceps.*

Hexapoda.

Thysanura.	Cinura.	LEPISMA SACCHARINA; *Malachis sp.*
	Collembola.	*Sminthurus sp.*
Odonata.	Libellulidæ.	*Erythemis simplicicollis; Lestes unguiculatus; Pantala flavescens; Sympetrum semicinctum.*
Isoptera.	Termitidæ.	*Termes sp.*
Corrodentia.	Psocidæ.	*Psocus sp.*
Mallophaga.	Liothoidæ.	*Menopon pallidum.*
Orthoptera.	Acrididæ.	*Dissosteria carolina.*
	Blattidæ.	*Periplaneta americana.*
	Gryllidæ.	*Gryllotalpa africana.*
Hemiptera.	Acanthiidæ.	*Cimex hirundinis;* CIMEX LECTULARIUS (Arkwright, Atkin, and Bacot[7]).
	Jassidæ.	*Aphrophora saratogensis; Gypona sp.; Ilburnia ipomœicola.*
	Pediculidæ.	*Gyropus gracilis; Hæmatopinus piliferus; Hæmatopinus suis;* PEDICULUS HUMANUS (da Rocha-Lima[1]).
	Pentatomidæ.	*Euschistus euschistoides; Œchila grisea.*
	Reduviidæ.	*Megalotomus quinquespinosus; Reduviolus sp.*
Neuroptera.	Chrysopidæ.	CHRYSOPA OCULATA.
Lepidoptera.	Lymantriidæ.	*Porthenia dispar.*
	Lycænidæ.	*Chrysophanus hypophlœus.*
	Pieridæ.	*Pieris rapæ.*
	Satyridæ.	*Satyrus alope.*
Diptera.	Asilidæ.	*Deromyia sp.; Promachus sp.*
	Culicidæ.	*Culex fatigans.*
	Hippoboscidæ.	MELOPHAGUS OVINUS Nöller.[15]
	Muscinæ.	*Hæmatobia serrata; Musca domestica.*
	Syrphidæ.	*Erystalis æneus; Erystalis arbustorum; Erystalis punctatus; Erystalis transversus.*
Siphonaptera.	Pulicidæ.	CTENOCEPHALUS CANIS; PULEX IRRITANS; *Spilopsyllus simplex.*
Coleoptera.	Alleculidæ.	*Isomera sericea.*
	Carabidæ.	*Anisodactylus agricola; Colcodiscus lucipatens; Cryptomorpha desjardinsii; Lebia sp.*
	Chrysomelidæ.	*Cryptocephalus sp.*
	Coccinellidæ.	*Adalia bipunctata.*
	Cicindelidæ.	*Cicindela punctulata.*
	Curculionidæ.	*Tychius picirostris.*
	Lamiidæ.	*Urographis fasciata.*
	Lampyridæ.	*Photinus sp.*
	Nitidulidæ.	*Pallodes pallidus.*
	Otiorhynchidæ.	*Brachyrhinus ovatus.*
	Scarabæidæ.	*Lachnosterna sp.; Macrodactylus subspinosus; Pelidnota punctata.*
	Staphylinidæ.	*Leistotropus cingulatus.*
	Telephoridæ.	*Malthodes parvulus; Telephorus lineola.*
	Tenebrionidæ.	*Gonocephalum seriatum.*

Hymenoptera.	Apidæ.	*Apis mellifica; Bombus vagans; Xylocarpa varipunctata.*
	Eumenidæ.	*Eumenes fraternus; Monobia quadridens.*
	Formicidæ.	*Camponotus sp.; Monomorium pharaonis; Tapinoma sessile.*
	Ichneumonidæ.	CASINARIA INFESTA; *Echthromorpha maculipennis.*
	Mutillidæ.	*Mutilla sp.*
	Pelecinidæ.	*Pelecinus polyturator.*
	Sphecidæ.	*Chlorion ichneumoneum.*
	Tenthredinæ.	*Arge erythrosoma; Arge sanguinea.*
	Vespidæ.	*Polistes pallipes; Vespa maculata.*

DISCUSSION.

From the observations it follows that the occasional occurrence of *Rickettsia* in certain species of the following groups may be accepted although these groups are not closely related in a phylogenetic sense: Attidæ, Trombidiidæ, Argasidæ, Ixodidæ, Cinura, Acanthiidæ, Pediculidæ, Hippoboscidæ, Chrysopidæ, Pulicidæ, and Ichneumonidæ. Systematically there is a sharp line of cleavage between the first four families, which are arachnids, and the other groups, which are insects. Among the latter, members of the suborder Cinura are considered to be very primitive in their affinities and as such may be contrasted with those belonging to the other families which comprise representatives of as many as six different orders. There are evident no common attributes in these groups which would explain the presence of *Rickettsia*.

The majority of the species which possess *Rickettsia* live entirely upon mammalian blood (*Amblyomma americana, Amblyomma hebræum, Boophilus decoloratus, Cimex lectularius, Ctenocephalus canis, Dermacentor variabilis, Dermacentor venustus, Margaropus annulatus, Margaropus annulatus australis, Ornithodoros turicata, Pediculus humanus, Pulex irritans, Rhipicephalus sanguineus*, and *Rhipicephalus evertsi*); but one is insectivorous (*Saltieus scenicus*); two are insectivorous in larval stages, becoming vegetarian in adult life (*Casinaria infesta* and *Chrysopa oculata*); one is chiefly vegetarian but will eat wool (*Lepisma saccharina*); and two are usually wholly vegetarian (*Atomus sp.* and *Lucoppia curviseta*). In view of these facts we can hardly go further than to say that although blood feeding may deter-

mine the presence of certain *Rickettsia*, it is not responsible for the occurrence of all of them.[17]

Neither is it possible to link the presence of *Rickettsia* with a recurring phase in the physiologic behavior of the several species. Some are mammalian ectoparasites, while others are free moving and winged. Only comparatively few species that contain *Rickettsia* are associated with disease. The influence of changes in season, of variations in moisture, of temperature, and of pressure must be determined by the close experimental study of individual forms, and is not within the scope of this paper.

The restriction of *Rickettsia* to certain tissues in some species (*cf. Dermacentor variabilis*, *Rhipicephalus sanguineus*, etc.) and their wide distribution throughout the body in others (*cf. Ctenocephalus canis* and *Cimex lectularius*) would seem to indicate either that the conditions determining their occurrence may be local or widespread or else that they themselves differ in their requirements. Their presence in the eggs of *Amblyomma americana*, *Amblyomma hebræum*, *Boophilus decoloratus*, *Dermacentor variabilis*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodoros turicata*, *Rhipicephalus sanguineus*, *Rhipicephalus evertsi*, and *Salticus scenicus* suggests not only that egg cells offer an unusually favorable medium for their growth and multiplication but also that their presence may be the result of direct passage to the offspring. The tendency which the *Rickettsia* exhibit to form definite and well defined clumps in the cytoplasm of egg cells is often even more pronounced than is illustrated in Figs. 1 and 10. It indicates that after penetration the organisms multiply *in loco* without further migration, for a time at any rate. Other favorable places for multiplication are found in the cells of the Malpighian tubules which frequently contain *Rickettsia*, like those inhabiting the

[17] Hindle's discovery (Hindle, E., *Parasitology*, 1921, xiii, 156) of organisms resembling *Rickettsia melophagi* within the intestinal lumen of 7 to 8 per cent of horse lice (*Trichodectes pilosus*) examined, is not of itself sufficient evidence to conclude that the infection is not acquired through the ingestion of infected blood. Although the Mallophaga have their mouth-parts adapted for biting rather than for sucking, and live chiefly upon hair, epidermal scales, etc., the possibility remains that they may occasionally take blood which has escaped from scratches or abrasions in the skin, unless it is definitely excluded through the failure of a microscopic examination of the intestinal contents to reveal any blood pigment.

egg cells in no less than eight of the above mentioned species. Cells of the intestinal epithelium contain *Rickettsia* of rather varied types in *Casinaria infesta*, *Ctenocephalus canis*, *Dermacentor venustus*, *Lepisma saccharina*, *Lucoppia curviseta*, and *Rhipicephalus evertsi;* while still other *Rickettsia* apparently remain free in the intestinal lumen or penetrate into different tissues in *Cimex lectularius*, *Pulex irritans*, and *Rhipicephalus evertsi*. But how they come to take up special positions removed from possible points of entry is not easily explained if the statement is true that, as a group, they possess no power of independent motility. Somewhat similar examples of the specific localization and multiplication of bacteria in mammalian cells not pronouncedly phagocytic are recorded by Smith[18] and Tyzzer.[19]

Notwithstanding their ability to live in nearly related forms, as, in the light of Wolbach's work, may be inferred from Maver's[20] success in transmitting the virus of Rocky Mountain spotted fever by means of *Dermacentor marginatus*, *Dermacentor variabilis*, and *Amblyomma americana*, they do exhibit a marked preference for certain hosts. The specificity seems to hold even for nearly related species of the same genus. Thus Arkwright, Atkin, and Bacot found *Cimex lectularius* to harbor one organism and *Cimex hirundinus* another, distinguishable in size, shape, and staining reaction. Reference to the observations will indicate how different is the content of organisms in *Amblyomma americana*, *Amblyomma hebræum*, *Amblyomma maculatum*, and *Amblyomma tuberculatum*. Similarly, within the genus *Dermacentor* there appears to be a somewhat parallel case of host specificity. Specimens of *Dermacentor venustus* from the States of Montana and Washington were found to contain apparently identical organisms; and examples of *Dermacentor variabilis* from Illinois and Maryland possessed similar organisms which differ from those just mentioned only in being somewhat more slender and less widely distributed through the tissues (Figs. 9 and 10). But the organisms cannot be definitely identified on purely morphologic grounds in the absence of cultural criteria. Differences in morphology may only reflect a different environment.

[18] Smith, T., *J. Exp. Med.*, 1919, xxix, 454.
[19] Tyzzer, E. E., *J. Med. Research*, 1917–18, xxxvii, 307.
[20] Maver, M. B., *J. Infect. Dis.*, 1911, viii, 327.

That the association of certain *Rickettsia* with definite insect hosts is a phenomenon of wide distribution geographically may be safely deduced from the observation that specimens of *Margaropus annulatus* from the states of North and South Carolina and Louisiana are uniformly infected with what would seem to be the same organism, while examples of *Margaropus annulatus australis* from the islands of Jamaica and Trinidad contain an organism which differs only in being considerably longer. A more striking instance of the same sort is afforded by *Cimex lectularius*. The work of Arkwright, Atkin, and Bacot, and of Bacot[21] has shown that a large percentage of bedbugs in both London and Warsaw are parasitized with *Rickettsia*. The existence of similar organisms (clearly identified by their distinctive morphology and distribution) in specimens captured in New York, the Hawaiian Islands, China, Jamaica, and Dutch Guiana as herein reported seems to point to an almost world-wide infestation. The failure to find the bodies in bedbugs from Antigua and Trinidad means little, since the preservation of the specimens from these localities was not wholly satisfactory. It is not clear how the bedbugs acquire the infestation unless it is from other bedbugs. Possibly they may transmit it to *Opsicœtus personatus* which regularly feeds upon them but which has not yet been studied in this connection.

In this survey lice taken in New York were found to be devoid of *Rickettsia*. The same is true of specimens from Peking, where, nevertheless, cases of typhus fever occasionally occur in the winter months. Specimens from Bermuda, Canton, Jamaica, and Antigua, as one would expect, failed to contain the bodies; but others from the Island of Trinidad contained some closely resembling *Rickettsia pediculi* and presumably therefore of a benign type.

A recognizable cellular reaction is usually absent as noted by Wolbach in his description of the parasite of Rocky Mountain spotted fever. The cells containing *Rickettsia* are sometimes greatly distended and occasionally rupture (Fig. 22), but otherwise there is no outspoken evidence of injury unless perhaps in the cases of *Dermacentor variabilis* and *Salticus scenicus*. In the former, the cytoplasm of the ovarian epithelial cells is very much more granular and the

[21] Bacot, cited by Wolbach, Todd, and Palfrey.[9]

nuclei show less uniformity in their staining reactions than in either *Dermacentor albipictus* or *Dermacentor venustus*. In *Salticus scenicus* the *Rickettsia*, when present in the egg cells, are clumped and surrounded by a capsule which must represent a reaction on the part of the cytoplasm (Fig. 19). One cyst of this type, about the size of the nucleus, but quite distinct from it in structure, may be found in every egg cell of infected specimens, but it is not clear why the cytoplasm of other cells containing apparently identical organisms does not respond in the same way (Figs. 18 and 20). In no instance was it possible to detect any preference on the part of any of the *Rickettsia* for special parts of the cytoplasm or for areas containing larger or smaller amounts of mitochondria. When they are present in the same cells with Gram-positive bacilli, as in the egg cells of *Amblyomma americana*, they do not intermingle with such organisms but tend to form separate clumps more peripherally situated.

It is not to be expected that all the phenomena observed in connection with *Rickettsia* will find their explanation in the same factors, because so many different organisms are grouped under this heading. The wide differences within the group become evident on a perusal of the literature. From being "smaller than the smallest bacteria" (*Rickettsia prowazeki*) they range to filaments several microns in length (*Rickettsia lectularia*). They may be coccal, diplococcal, streptococcal, or bacillary. They may be homogeneous, show polar staining like some bacteria, or exhibit a differentiation into materials capable of being colored red and blue during division. Some are cultivable,[22] while others are resistant, some are typically extracellular, others intracellular. And finally their dispersal in the tissues of the host, and their life histories, exhibit few if any features in common. The parasite of Rocky Mountain spotted fever, as described by Wolbach, seems to stand alone in its ability to lead, for a time at least, an intranuclear existence; and in certain other features of its life history is unique. No organism has yet been reported, except perhaps *Rickettsia hirundinus*, which even approximates in its behavior to *Rickettsia lectularia*, and a glance at the plates will be

[22] *Rickettsia melophagi*[15] and *Rickettsia Rocha-Limæ* (Weigl, R., *Przeglad Epidemjol.*, 1921, i, 373).

sufficient to indicate the variability of the intracellular Gram-negative *Rickettsia* herein described.

From the foregoing discussion it will be seen that evidence is not lacking that the bodies called *Rickettsia* found in the tissues of insects and arachnids are true microorganisms. Indeed, it is not easy to refer to them by the lifeless non-committal term of "bodies." They occur in large numbers and no special methods are required for their demonstration. Severe pathological changes do not, as in mammals, render their demonstration difficult.

Woodcock has claimed that the bodies referred to as *Rickettsia* in human and mammalian tissues are nothing but phagocytosed red blood cells, and has only recently extended this interpretation to *Rickettsia* in insects.[23] The resemblance in shape between granules of pigment of hematogenous origin and certain *Rickettsia* in insects is undeniable, but the pigment granules usually retain some trace of yellow color and are never typically basophilic. Furthermore, *Rickettsia* are frequently found in tissues which do not exhibit the power of phagocytosis, or possess it only in a restricted degree.

Although they closely resemble mitochondria morphologically, as the plates show, confusion of the two is unnecessary. A comparison of Figs. 1 and 13, of *Rickettsia* in egg and nephridial cells, with Figs. 2 and 4, illustrating mitochondria in the same locations, will reveal the fact that the latter exhibit more pronounced pleomorphism, and that, being normal cell constituents, they are met with in all cells. Mitochondria never occur in the intestinal lumen or apart from cells as do *Rickettsia*, neither do they stain with basic dyes, except under special conditions, and then only faintly. Additional distinguishing points have been given elsewhere by Cowdry and Olitsky.[24] That the organism of Rocky Mountain spotted fever is independent of mitochondria in mammalian tissues has been shown by Nicholson.[25]

Tinctorially, *Rickettsia* more closely approximate to nuclear material than to mitochondria, but all my attempts to establish a genetic relation between the two have broken down. The extrusion

[23] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1921, xxxvii, 418; 1922, xxxix, 251.

[24] Cowdry, E. V., and Olitsky, P. K., *J. Exp. Med.*, 1922, xxxvi, 521.

[25] Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

of nuclear substance is marked in the egg cells of *Amblyomma americana*, but as Figs. 3 and 1 show, it bears no resemblance to the *Rickettsia* bodies in the eggs of the same species, the property of taking up basic dyes being probably due, as in the case of some bacteria, to the possession of a relatively large amount of diffusely distributed iron-containing protein.

The conclusion seems almost unavoidable that some at least of the intracellular Gram-negative *Rickettsia* described in this paper are merely bacteria, but Wolbach's discovery that the parasite of Rocky Mountain spotted fever passes through a definite series of morphologic changes after its entry into ticks, occupying a period of 2 or 3 weeks duration, illustrates the need for caution in any general conclusion. The possibility must be entertained that other *Rickettsia* may also exhibit a more or less complicated life history, no hint of which would be given by the examination of tissues at one stage only, because the behavior of the organisms in the preceding days and in the weeks to follow could not be safely predicted. In other words, individual observations may offer only cross-sectional views of life histories which are in reality complicated and involved. One hypothesis, however, is capable of being partly tested cytologically; namely, the suggestion advanced by Jungmann and others that *Rickettsia* are of chlamydozoal nature. Literally translated the term means "mantle animals" and is intended to indicate that at a certain stage in their life history the "animals" penetrate into cells and are there surrounded by a mantle or cloak of material produced by the cell in response to their action. The cloak is said to be composed of nuclear substance and according to Lipschütz[26] may be detected microscopically in the case of certain Chlamydozoa. But careful search has revealed the fact that the organisms herein described stain homogeneously in each instance and show no evidence of being covered with a substance of a different nature.

SUMMARY.

In the absence of a satisfactory definition of *Rickettsia* the observations herein recorded were arbitrarily limited to bacterium-like organisms which are intracellular and Gram-negative.

[26] Lipschütz, B., *Zentr. Haut- u. Geschlechtskrankh.*, 1921–22, iii, 3.

Rickettsia of this type were found in the following species: *Amblyomma americana*, *Amblyomma hebræum*, *Boophilus decoloratus*, *Atomus sp.*, *Casinaria infesta*, *Chrysopa oculata*, *Ctenocephalus canis*, *Dermacentor variabilis*, *Lepisma saccharina*, *Lucoppia curviseta*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Ornithodoros turicata*, *Pulex irritans*, *Rhipicephalus sanguineus*, *Rhipicephalus evertsi*, and *Salticus scenicus*. Since intracellular, Gram-negative *Rickettsia* have been recorded in the literature as existing in *Cimex lectularius*, *Dermacentor venustus*, *Melophagus ovinus*, and *Pediculus humanus*, the occasional occurrence of such bodies must be conceded in the following groups not closely related phylogenetically: Attidæ, Trombidiidæ, Argasidæ, Ixodidæ, Cinura, Acanthiidæ, Pediculidæ, Hippoboscidæ, Chrysopidæ, Pulicidæ, and Ichneumonidæ.

The species which harbor *Rickettsia* differ widely in diet and habitat. One such species is insectivorous throughout life, two are insectivorous in larval stages, becoming vegetarian in the adult condition, one is chiefly vegetarian but partakes of some animal products, and two are usually entirely vegetarian; while the remainder subsist wholly upon a diet of mammalian blood. *Rickettsia* are associated, in only a few cases, with diseases in mammals.

The evidence at hand does not lead beyond the conclusion that the *Rickettsia* mentioned above are true Gram-negative microorganisms, easily distinguishable from mitochondria and all other cytoplasmic and nuclear granulations, rather completely adapted to an intracellular existence, exhibiting in some cases a remarkable degree of host specificity, and often inherited through the eggs.

EXPLANATION OF PLATES.

All the drawings were made with a Zeiss apochromatic objective 1.5 mm., compensating ocular 6, and camera lucida. Unless otherwise noted, the specimens were fixed in 10 per cent formalin, followed by Regaud's fluid, and were colored with Giemsa's stain. Magnification, × 1,500.

PLATE 25.

FIG. 1. *Amblyomma americana*. Egg cell, fixed in Regaud's fluid and colored with Giemsa's stain, containing small Gram-positive organisms deeply stained, and larger Gram-negative *Rickettsia* lightly stained and clumped near the cell membrane.

FIG. 2. The same, fixed in the same way and stained with iron-hematoxylin to show the morphology and arrangement of the mitochondria.

FIG. 3. The same, fixed in Bouin's fluid and stained with iron-hematoxylin to show the distribution of the amorphous nuclear emanations.

FIG. 4. *Amblyomma americana.* Malpighian tubule, fixed in Regaud's fluid and stained with iron-hematoxylin to illustrate the characteristic arrangement of mitochondria. Compare them with *Rickettsia* in similar cells of other species (Figs. 8, 9, 13, and 22).

FIG. 5. *Atomus sp.* Large green pigmented hypoblastic cell containing *Rickettsia.*

FIG. 6. *Chrysopa oculata.* Fat cells containing *Rickettsia.*

PLATE 26.

FIG. 7. *Casinaria infesta.* Intestinal epithelial cells containing *Rickettsia.*

FIG. 8. *Ctenocephalus canis.* Malpighian tubule containing *Rickettsia.*

FIG. 9. *Dermacentor variabilis.* Malpighian tubule cells containing *Rickettsia* which are much finer and more thread-like than those represented in Figs. 13 and 22.

FIG. 10. *Dermacentor variabilis.* An egg cell.

FIG. 11. *Lepisma saccharina.* Intestinal epithelial cells containing *Rickettsia.*

FIG. 12. *Lucoppia curviseta.* Large pigmented intestinal epithelial cell with *Rickettsia.*

FIG. 13. *Margaropus annulatus.* Malpighian tubule cells with large *Rickettsia* which may be contrasted with the mitochondria illustrated in Fig. 4.

FIG. 14. *Margaropus annulatus.* Part of an egg cell.

PLATE 27.

FIG. 15. *Margaropus annulatus.* Part of an older egg cell with well developed yolk spherules.

FIG. 16. *Ornithodoros turicata.* Epithelial cell of rectal sac containing *Rickettsia.*

FIG. 17. *Pulex irritans.* Fat cell containing *Rickettsia.*

FIG. 18. *Salticus scenicus.* Above a connective tissue cell, and below a cross-section of a muscle cell, both containing *Rickettsia.*

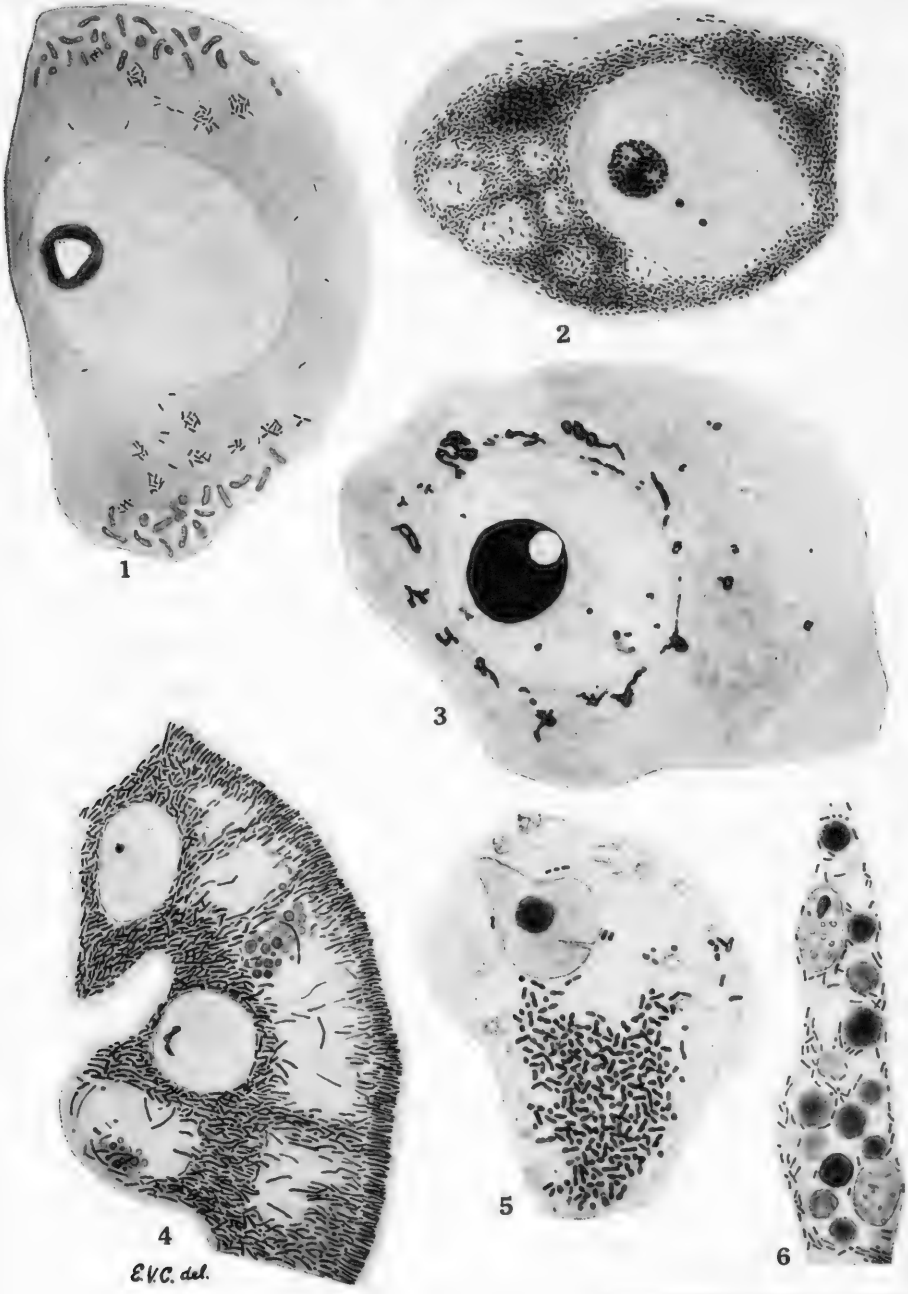
FIG. 19. *Salticus scenicus. Rickettsia* encysted within the cytoplasm of an egg shell.

FIG. 20. *Salticus scenicus. Rickettsia* within a nerve cell.

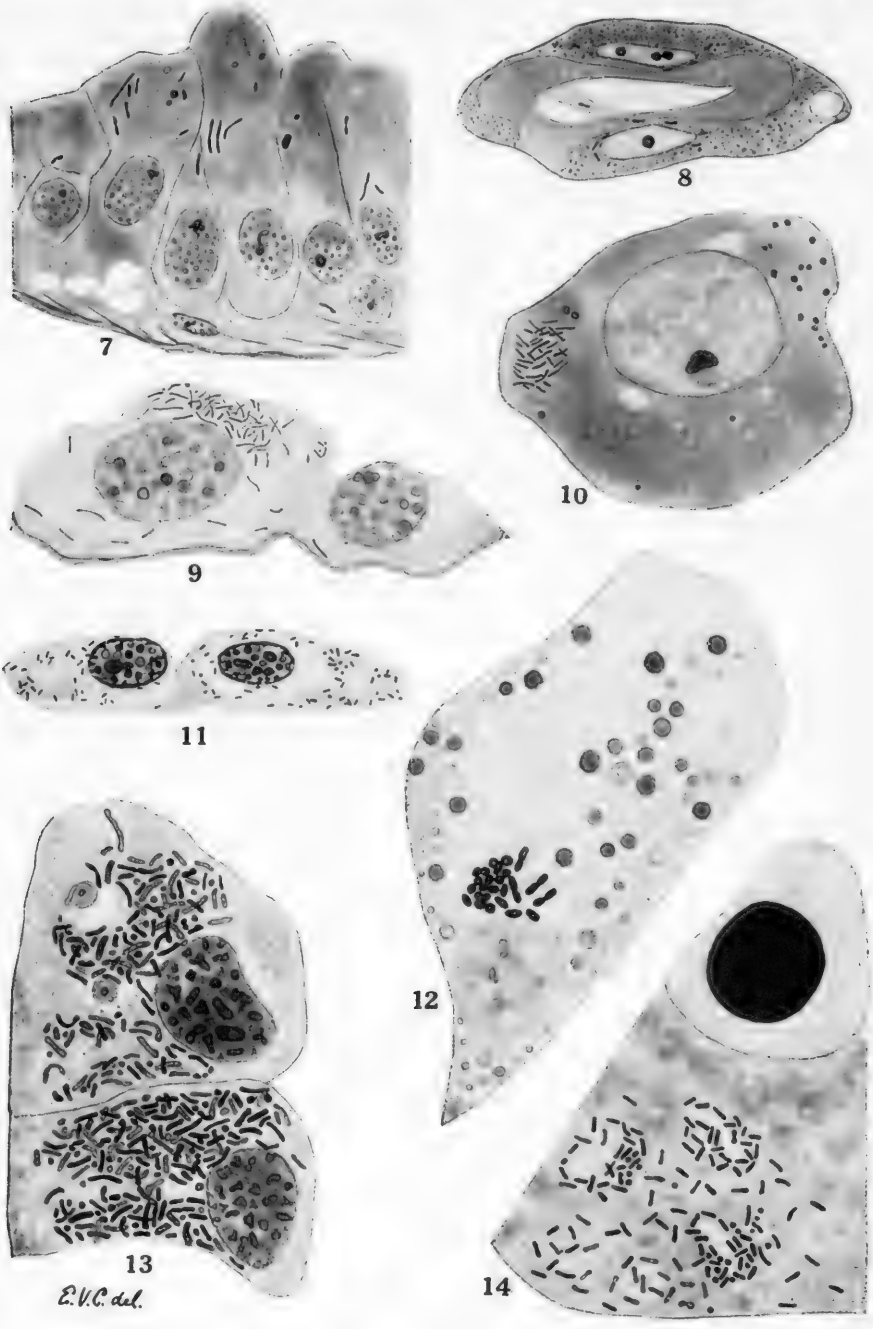
FIG. 21. *Salticus scenicus. Rickettsia* between nerve fibers.

FIG. 22. *Rhipicephalus sanguineus.* Portion of a Malpighian tubule with the lumen containing many *Rickettsia.*

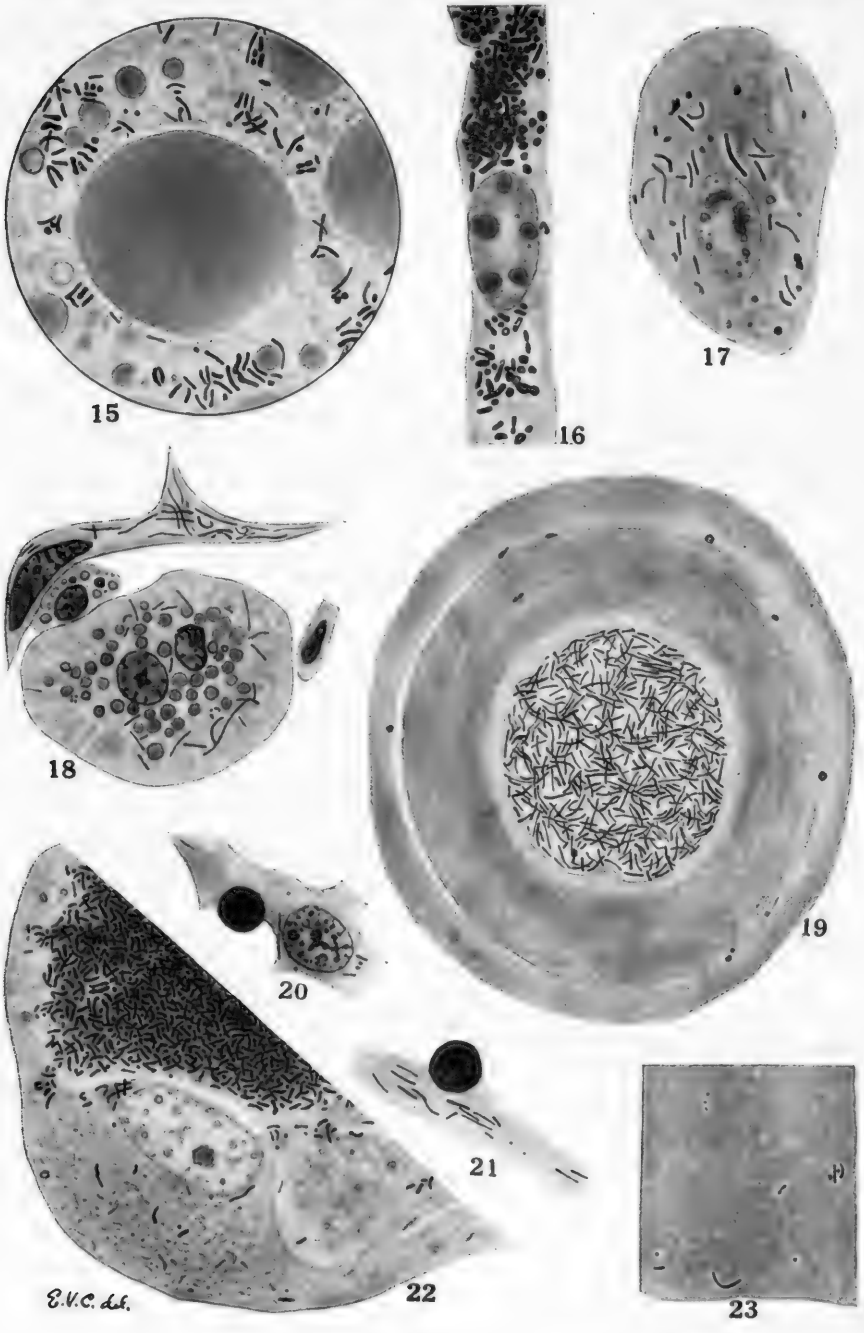
FIG. 23. *Rhipicephalus sanguineus.* Part of the cytoplasm of an egg cell containing similar *Rickettsia.*



(Cowdry: *Rickettsia* in insects and arachnids.)



(Cowdry: *Rickettsia* in insects and arachnids.)



(Cowdry: *Rickettsia* in insects and arachnids.)

THE DETERMINATION OF LUNG VOLUME WITHOUT FORCED BREATHING.

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(Received for publication, November 6, 1922.)

The dilution method for determining lung volume invented by Davy (1) and modified by Gréhant (2), by Bohr and his coworkers (3), and recently by Lundsgaard and Van Slyke (4) rests on the principle of mixing the air in the lungs with a known volume of foreign gas (H_2 or O_2) and calculating the air content of the lungs from the extent to which either the foreign gas or the nitrogen of the lung air is diluted. This method yields satisfactory results when subjects can breathe deeply, so that five or six respirations cause complete mixture of the lung air with the diluting gas. When the subject, however, because of weakness or respiratory disturbance, cannot greatly increase the depth of his respirations, so much time is required for complete mixture that volume changes, due chiefly to the difference between evolution of carbon dioxide and absorption of oxygen, make accurate results unobtainable. In an attempt to study the lung volumes in cases of heart failure this difficulty was encountered. While constant lung volumes could be obtained in a given subject with a fair degree of accuracy when he rebreathed O_2 for a certain length of time, prolongation of the time of rebreathing invariably increased the observed lung volume. Similar inaccuracies and sources of error have been observed by previous investigators.

Bruns (5), using the method of Bohr and Hasselbalch, observed that in the normal subject six breaths of a depth of 1 liter were sufficient to accomplish complete mixture, whereas six breaths of 500 cc. each were insufficient. But he noted that in subjects suffering from emphysema and heart disease with stasis in the lesser circulation, unlike the normal subject, six breaths of 1 liter each did not establish equilibrium between the gas mixture in the lung and the spirometer. Bruns attributed this to pathological changes in the lungs with disturbances in the normal processes of gaseous diffusion, and concluded by questioning the value

of the method of Bohr and Hasselbalch for determining lung volumes in cases of emphysema and heart disease. Siebeck (6) too observed this difficulty and regarded his own values for mid-capacities in cases of heart disease as very uncertain and probably too low because of inadequate gas mixture. Lundsgaard and Schierbeck (7) have recently published a systematic study of the number and depth of respirations required to secure full mixture with volumes of air contained in the lungs.

They found that if the air left in the lungs were 1.3 liters three, five, and seven respirations were necessary to secure mixture with a depth respectively of 2 1.5, and 1 liters. If the air left in the lungs were increased to 2 liters, as in emphysematous patients, four, six, and nine respirations of similar depth were required. As they increased the amount of air left in the lungs the number of respirations required increased. They therefore concluded that a vital capacity below 1 liter, as may occur in cases of heart disease, makes it difficult to obtain full mixture with the usual dilution method.

The need was felt, therefore, to devise a method which would obviate these difficulties and satisfy the following desiderata: (1) to provide for complete mixture between the foreign gas and the lung air without forced deep breathing; (2) to give constant values independent of the length of time during which the subject rebreathes the gas after mixture is once established; and (3) to supply values for all lung volumes, *i.e.* total capacity and its subdivisions, coincidently.

Principle of the Method.

The calculation of lung volume was based not upon the dilution of one gas but upon the volume ratio of two gases. The subject breathed to and from a spirometer containing oxygen sufficient to satisfy his requirements for the length of the experiment. Added to the oxygen was a known volume of hydrogen, approximating the volume of nitrogen in the lungs. The CO_2 was continuously removed by a scrubber of sodium hydroxide shells. After mixture was complete a sample of the mixed gases was taken from which the O_2 and remaining traces of CO_2 were removed by absorption with potassium pyrogallate. The ratio of H_2 to N_2 in the residue was then determined. From this ratio and the known volume of H_2 the volume of N_2 (originating from the lung air) was calculated as

$$\text{Vol. } N_2 = \text{Vol. } H_2 \times \left(\frac{\text{Vol. } N_2 \text{ in sample}}{\text{Vol. } H_2 \text{ in sample}}\right)$$

The volume of N_2 in the air of the lungs having thus been obtained, the lung volume could be calculated:

$$\text{Lung volume} = \frac{\text{Vol. } N_2}{0.791} \text{ or } 1.264 \times \text{Vol. } N_2$$

since the nitrogen constitutes 0.791 of the air.

Method, Technique, and Apparatus.

For determining the total capacity of the lungs a combination method similar to Bohr's, consisting partly of direct spirometry (for vital capacity) and partly of gas mixture (for residual air), was used. The spirometer was a freely movable, well counterpoised, cylindrical one with a water seal having a capacity of 6.5 liters. It was equipped with a thermometer and a side arm bearing a writing pen to record its movements on a slowly revolving drum.

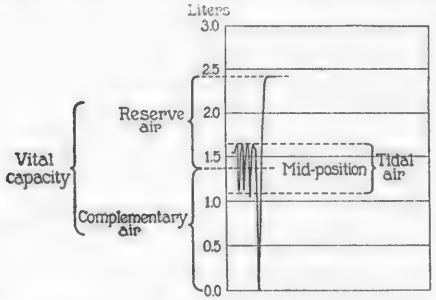
The subject to be investigated was connected to the apparatus by means of a rubber mouthpiece and five-way stop-cock. His nostrils were clamped with a nose clip.[1] The subject was allowed to breathe room air, the expired air if desired being collected in a Tissot spirometer for the purpose of measuring the minute volume of pulmonary ventilation and the tidal air. Then, without his knowledge, he was connected with the 6.5 liter vital capacity spirometer. He continued quiet, normal breathing into and from the spirometer for three or four breaths, when he was urged to take the deepest possible inspiration and then to expire completely.

By the use of a kymograph a graphic record was obtained by which the vital capacity and its constituent portions could be directly measured with a rule calibrated in the same units as the spirometer (Text-fig. 1).

At the point of complete expiration, communication between the subject's lungs and the spirometer was cut off by turning the cock

[1] Bohr's objection to the mouthpiece and preference for a mask have not been substantiated by our experience. Some patients prefer one, some the other. The mouthpiece is more readily rendered leak-tight and in dealing with as rapidly diffusible a gas as hydrogen this is an important consideration. Siebeck found no difference in lung volume determinations when using masks or mouthpieces, but preferred the latter because of the smaller dead space.

which permitted him to breathe to and from a third spirometer containing a known gas mixture of H_2 and O_2. He breathed in and out of this spirometer through a circulation device and scrubber which removed CO_2. The apparatus is seen in Text-fig. 2. In an earlier communication (8) the writers described its use with a rubber bag to hold the H_2-O_2 mixture. The bag was later abandoned and replaced by a specially designed spirometer, since diffusion of H_2 through the rubber introduced in the longer experiments a considerable and disturbing source of error. The spirometer was of the cylindrical type used by Peabody for the study of vital capacities and manufactured by Sanborn Company, Boston. It was modified by closing the top of the inner chamber so that when the bell was lowered



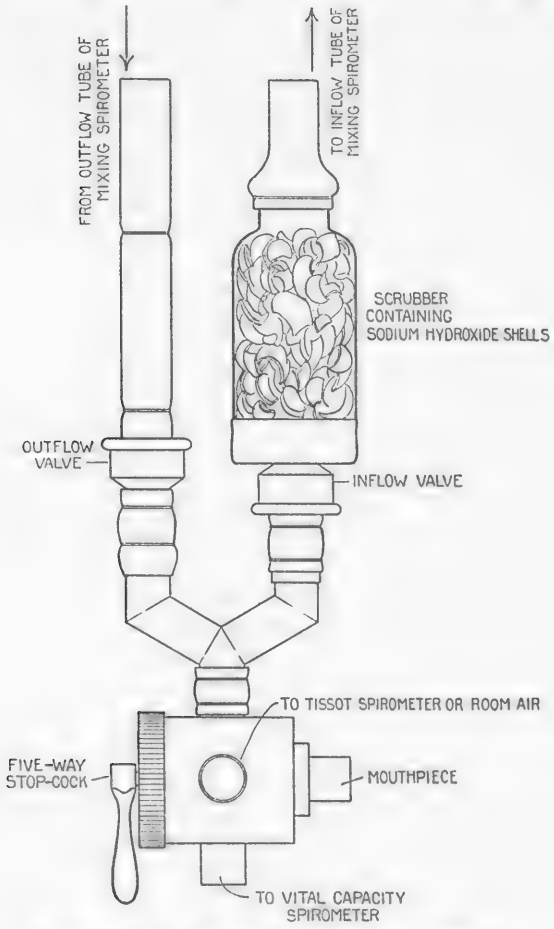
TEXT-FIG. 1. Graphic record of the vital capacity.

its interior was almost completely occupied by a solid center core. This core was pierced by two tubes instead of one as in the ordinary spirometer, so that with an appropriate arrangement of valves the subject might expire into the spirometer through one tube and inspire from the spirometer through the other. Every breath, therefore, tended to mix the air in the system. With such an arrangement no mixing fan was found necessary.[2]

The mixing spirometer was prepared for use by washing out with O_2 from a pressure cylinder. Three washings of 6 to 7 liters were sufficient to sweep out any residual N_2 or H_2 from a preceding obser-

[2] Roth (Roth, P., *Boston Med. and Surg. J.*, 1922, clxxxvi, 457) has shown that with the Benedict new portable type of metabolism spirometer valves may be used to advantage over the electric impeller commonly employed.

vation. Enough O_2 was left in the spirometer so that after H_2 was added the total gas volume equaled or slightly exceeded the patient's



TEXT-FIG. 2. Stop-cock, valves, and scrubber.

vital capacity. With this provision the first inspiration from the spirometer after the patient had forcibly expelled all the air from his lungs did not completely empty the spirometer, which would

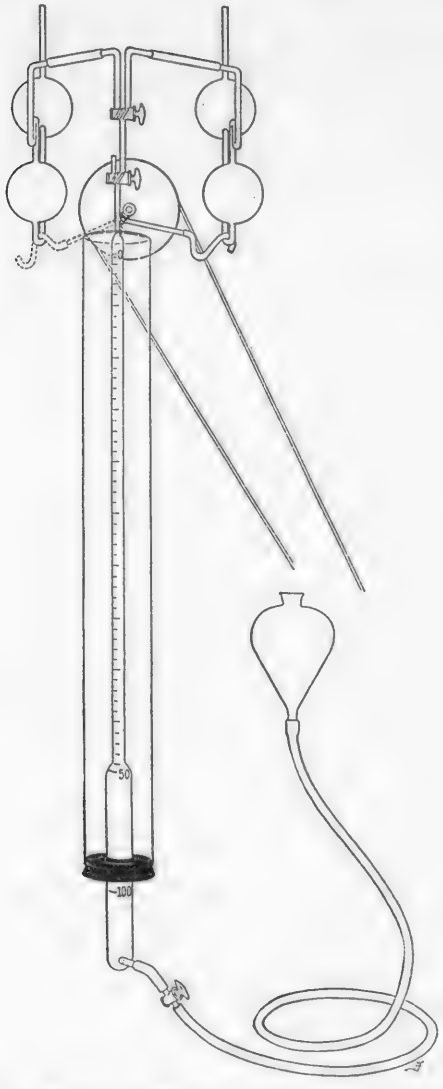
have embarrassed respiration. The volume of O_2 in the spirometer was likewise sufficient to support comfortable respiration for 5 to 8 minutes. From 2 to 4 liters were used in the present study. It was desirable to use a volume of H_2 which equaled approximately the anticipated volume of N_2 in the lungs. This provided for a ratio of $\frac{N_2}{H_2}$ approximating 1 and thereby minimized the effect of analytical error. We have used electrolytically prepared H_2 supplied in cylinders by the International Oxygen Company, Newark. With H_2 so prepared we have never encountered contamination with arsine, which may be a source of danger when H_2 is produced from the action of acid on zinc.

Method of Gas Analysis.

The gas analysis was done in a burette enclosed in a water jacket. The burette, by means of a three-way glass stop-cock and capillary tubing, communicated with two Hempel pipettes, such as are used in the gasometric amino nitrogen determination (9). One contained potassium pyrogallate for the absorption of O_2 and CO_2, the other contained a solution containing 5 gm. of sodium picrate and 2 gm. of colloidal palladium per 100 cc. This solution served for the quantitative removal of H_2. Both Hempel pipettes were shaken mechanically by an eccentric driven by a small motor. The burette and Hempel pipettes are shown in Text-fig. 3.

About 60 cc. of gas mixture were introduced into the burette from one of the sampling bulbs. This volume was not measured accurately, as O_2 and CO_2 do not enter into the final calculation. The gas mixture was driven over into the Hempel pipette containing potassium pyrogallate. The pipette was shaken vigorously for about 5 minutes, when the residual gas, consisting of hydrogen and nitrogen, was run back into the burette and measured.

The hydrogen analysis was accomplished by the method described first by Paal and Hartmann (10) and modified by Brunck (11). It depends upon the reduction of picric acid in the presence of colloidal palladium to triaminophenol according to the equation $C_6H_2(OH)(NO_2)_3 + 9H_2 = C_6H_2(OH)(NH_2)_3 + 6\ H_2O$. Picric acid was used in the form of its sodium salt.



TEXT-FIG. 3. Burette and Hempel pipettes used in the gas analysis.

5 gm. of picric acid were neutralized with 22 cc. of N NaOH. Solution was completed by warming and adding water up to about 50 cc. To this solution was added a suspension of 2 gm. of colloidal palladium (Merck) in H_2O. The final volume was 100 cc. This mixture should theoretically bind 4.37 liters of H_2 (at 0°C. and 760 mm. Hg.). The palladium could be recovered for use after the sodium picrate became inactive by acidifying with very dilute H_2SO_4. This precipitated the palladium which could be suspended in H_2O and redissolved by addition of dilute NaOH drop by drop.

According to Brunck the picrate-palladium method of hydrogen analysis is as accurate as the combustion method. We have found 20 to 30 minutes vigorous shaking to be sufficient for complete removal of H_2.

Calculation of Dead Space in the Mixing Spirometer and Five-Way Stop-Cock.

Since the mixing spirometer and circulation device formed a closed circuit when the five-way stop-cock was shut, the system could be filled with O_2 and washed free from residual N_2 and H_2. The stop-cock itself contained a small volume of air, and a certain percentage of air contamination was found in the purest samples of commercially supplied O_2 and H_2. To correct the error introduced by this small amount of air it was found best to determine its volume under the conditions of the experiment rather than to use assumed air corrections for the given gas volumes. Control experiments were performed with a known volume of air contained in a volumetric bulb[3] which was connected with the mixing spirometer through the five-way stop-cock. The bulb was used to replace the subject's lungs and a check on the method was thereby given as well as a constant correction to be applied to all lung volume determinations.

After repeated mixing of a liter of air in the bulb with the H_2-O_2 mixture in the spirometer, successive samples were taken from the spirometer in mercury sampling bottles. These samples were analyzed for their content of N_2 and H_2. The ratio of $\frac{N_2}{H_2}$ was ascertained

[3] The volumetric bulb was a spherical glass bulb opening at opposite points into two slender tubular necks, as on a flask. The bulb was calibrated to contain 1 liter between marks on the two necks.

for each sample, and, if constant, the mixture was known to be a complete one and to represent an equilibrium of all the gases in the system.

The analytical results of such successive sampling are shown below as well as the method for calculating the volume of air enclosed in the bulb (or lungs). An average discrepancy of 80 cc. was observed between the known volume of air in the bulb and the volume calculated on the basis of the $\frac{N_2}{H_2}$ ratio. This represented air present in the apparatus after washing out with O_2. In seven separate determinations this discrepancy varied by not more than 20 cc. To the average correction of 80 cc. 10 cc. were added representing the air enclosed in the mouthpiece. This figure then, 90 cc., was deducted from all lung volume determinations as a constant correction for air contained in the stop-cock and contaminations of air in the gas mixtures used.

Calculation of Residual Air.

An example is given of the calculation of the residual air from the analytical data.

Subject 1.—

Contents of mixing spirometer: O_2 3 liters.
H_2 1.5 liters.

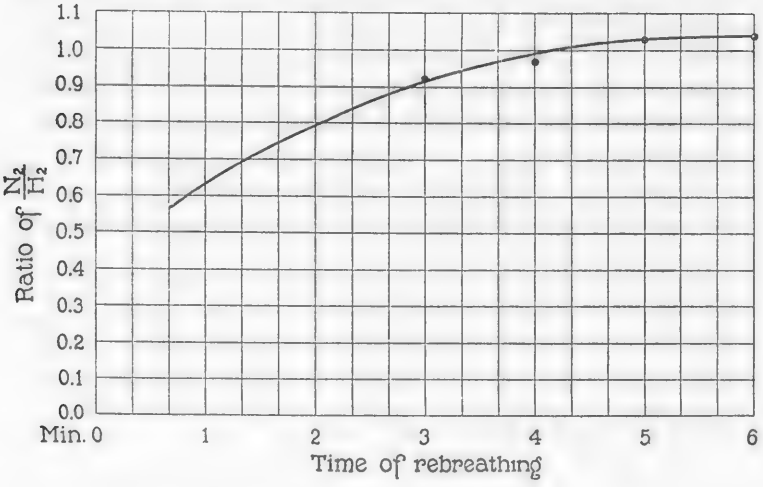
Sample taken after rebreathing 3 minutes.
Analysis: Gas sample 60 cc.
After K pyrogallate absorption 30.81 cc. $N_2 + H_2$
After picrate-palladium $\left.\begin{matrix}14.74 \text{ cc. } N_2 \\ 16.07 \text{ cc. } H_2\end{matrix}\right\} \frac{N_2}{H_2} = 0.917$

Sample taken after rebreathing 4 minutes.
Analysis: Gas sample 60 cc.
After K pyrogallate absorption 32.80 cc. $N_2 + H_2$
After picrate-palladium $\left.\begin{matrix}16.13 \text{ cc. } N_2 \\ 16.67 \text{ cc. } H_2\end{matrix}\right\} \frac{N_2}{H_2} = 0.966$

Sample taken after rebreathing 5 minutes.
Analysis: Gas sample 60 cc.
After K pyrogallate absorption 35.01 cc. $N_2 + H_2$
After picrate-palladium $\left.\begin{matrix}17.73 \text{ cc. } N_2 \\ 17.28 \text{ cc. } H_2\end{matrix}\right\} \frac{N_2}{H_2} = 1.03$

Sample taken after rebreathing 6 minutes.
Analysis: Gas sample 60 cc.
After K pyrogallate absorption 31.62 cc. $N_2 + H_2$
After picrate-palladium $\left.\begin{matrix}16.13 \text{ cc. } N_2 \\ 15.49 \text{ cc. } H_2\end{matrix}\right\} \frac{N_2}{H_2} = 1.04$

From the $\frac{N_2}{H_2}$ ratios a curve may be plotted with the ratios as ordinates and time in minutes of rebreathing as abscissæ. The curve drawn from the data above is shown in Text-fig. 4. Somewhere between 4 and 6 minutes the curve tended to become horizontal and the $\frac{N_2}{H_2}$ ratio constant. In this region the gases in the mixing spirometer and the air in the lungs were in equilibrium, complete mixture having occurred. The slight upward inclination of the curve after it has

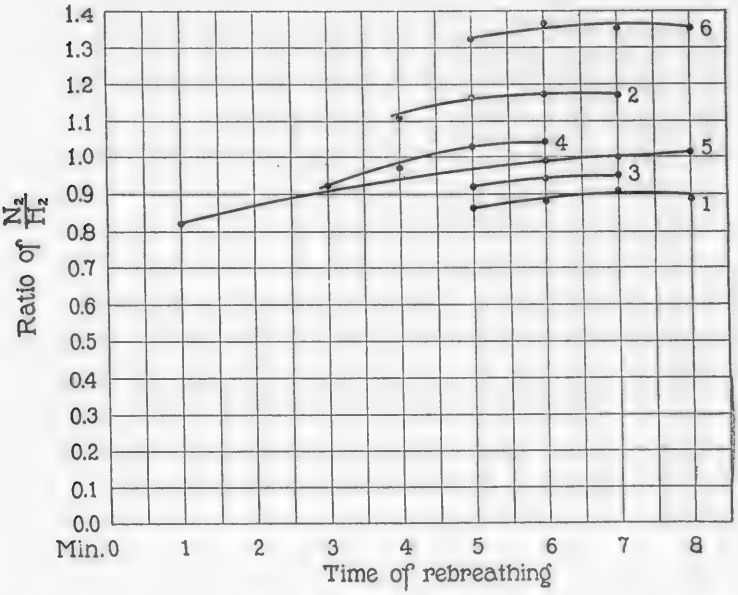


TEXT-FIG. 4. Curve showing the ratio of $\frac{N_2}{H_2}$ in Subject 1.

approached the horizontal position may be interpreted as being due to the continued elimination of nitrogen from the tissues into the gas mixture and to the absorption of hydrogen by the tissues. This would, of course, increase the N_2 and decrease the H_2 of the mixture and thus increase the ratio. An error of an indeterminable nature is introduced by this phenomenon. It is obvious that it must operate chiefly at the start of rebreathing when the partial pressure of hydrogen is relatively high and the partial pressure of nitrogen relatively low. Because of the low solubility of H_2 and the low tension of N_2

in the blood it may be assumed that the magnitude of this error is insignificant as compared with other sources of error which are discussed below.

To correct in a measure for this error, lung volume was calculated from that point in the curve where there was definite change in its



TEXT-FIG. 5. Curves 1 to 4 are from patients with cardiac disease, Curves 5 and 6 from normal subjects. In each case the lung volume was determined by multiplying the final $\frac{N_2}{H_2}$ ratio by the volume of H_2 in the mixing spirometer, and by 1.264, and deducting the constant correction.

direction. From the inspection of a series of curves (Text-fig. 5) mixture was assumed to be complete at that point where the difference in ratios was not greater than 0.02. In an $\frac{N_2}{H_2}$ ratio of 1, this would approximate the empirical error determined above for an artificial system in which no absorption or elimination occurred. The

lung volume (in this case residual air) was then calculated from the $\frac{N_2}{H_2}$ ratio thus:

1.03	×	1.264	×	1,500	=	1.95
Final $\frac{N_2}{H_2}$ ratio.		$\frac{1}{0.791}$		Vol. H_2		Air content of lungs, uncorrected.

From the 1.95 liters of calculated pulmonary air the 0.09 liter correction for dead space and gas impurities was deducted, leaving a corrected lung volume of 1.86 liters.

Text-fig. 5 shows a group of curves obtained from four cases of cardiac disease and two normal subjects. The length of time necessary to obtain complete mixture depended on a variety of factors such as depth and rate of pulmonary ventilation and the relative sizes of the volume of air enclosed in the lungs and of the volume of gases in the mixing spirometer. Besides the length and bore of the tubing through which the subject breathed, which must be taken into account, it is possible that factors other than these enter into the determination of the length of time needed for complete mixture, factors such as structural changes in the lungs and pooling of the air in emphysematous areas. Since each curve represents a somewhat different set of conditions one cannot generalize from them as to the length of time required to reach equilibrium.

Sources of Error.

In lung volume determinations a chief source of error lies in the inconstancy of the subject's lung position at the time mixture with the foreign gas is begun. Even with a trained normal subject variations in vital capacity of 100 cc. are not excessive and such variations may reflect themselves in the residual air and total lung capacity. Lack of training and the ready fatigability of the patient with cardiac disease makes it still more difficult for him to reach the point of complete inspiration or expiration with any degree of constancy. Such a source of error varies with the individual subject and is indeterminable.

Besides this source of error, there are certain errors inherent in the method. Since these may be combined in their effect and are

not easy to evaluate separately their combined effect was determined by control experiments in which a vessel containing known volumes of air was substituted for the lungs. In such determinations it was found that variations of ± 20 cc. in 1 liter occurred, making the error approximately 2 per cent.

Variations in the percentage of nitrogen of the lung air must be taken into account in a discussion of sources of error. Lundsgaard and Van Slyke (4) found in thirty-six individuals the extremes to be 78.4 and 79.6 per cent, though in twenty-seven of these they were 78.7 and 79.5 per cent. The effect of such variation would, of course, be a change in the $\frac{N_2}{H_2}$ ratio. Assuming a ratio of 1, the error introduced by the extreme variation would be ± 0.8 per cent. Addition of this error to that discussed above gives a total of 2.8 per cent.

SUMMARY.

1. A method is described for the determination of lung volumes without forced breathing.

2. The method determined total capacity and its subdivisions coincidently.

3. The values obtained were independent of the length of time during which the subject rebreathed after gas mixture was once established.

4. The maximum error of the method is estimated at ± 3 per cent of the lung volume determined. Considerably greater variation from the mean may be encountered, however, because of inconstancy in the lung positions assumed by the subject.

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EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

XII. The Effects of Subcutaneous Injections of Vaccines of Bacterium pneumosintes in Man.*

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(Received for publication, November 15, 1922.)

INTRODUCTION.

When in the course of our studies on *Bacterium pneumosintes* it was found that experimental infection protected rabbits against subsequent intratracheal inoculations of living organisms,[1] and that intravenous doses of living or dead bacteria stimulated specific antibody formation,[2] we were led to investigate the protective efficacy of small subcutaneous injections of the heat-killed organisms. The results of these experiments have already been reported in Paper X[3] of this series. They may be reviewed briefly as an introduction to the somewhat similar study in man which forms the basis of this article.

Three measured doses of heat-killed *Bacterium pneumosintes*, injected subcutaneously in rabbits at suitable intervals, induced an appreciable specific agglutinin formation in all but a few of the animals. The vaccinated rabbits were then found to be resistant to intratracheal injections of living *B. pneumosintes*, obtained from cultures or contained in the lung tissues of previously affected

* The results to be reported in this paper are based on a cooperative study with Lieutenant-Colonel Charles F. Craig, Medical Corps, U. S. Army, Director of the Laboratories of the Army Medical School at Washington, D. C. It was at his invitation that this work was begun and his keen interest and cooperation made the study possible. Our thanks are also due to the members of his staff for most cordial and helpful assistance.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 685.

rabbits, in doses pathogenic for normal animals. Consequently, in the absence of the damage which *B. pneumosintes* induces in unprotected rabbit lungs, these vaccinated animals, after intratracheal injection with living *B. pneumosintes*, were found to be as a rule as resistant as normal rabbits to secondary infection with pneumococcus, streptococcus, and *B. pfeifferi* under conditions in which control rabbits were affected. This resistance to infection with ordinary bacteria was maintained also during the period immediately following a single injection of killed *B. pneumosintes* five times the usual dose. Thus no evidence indicative of a so called "negative phase" was obtained. Attention is directed to these observations because of the association of pulmonary injury due to *B. pneumosintes* with secondary invasions of the damaged lung by ordinary bacteria[4] which parallels so nearly the relation of epidemic influenza to secondary pneumonic infections in human beings.

The conclusion stated was that "the mildness of the local reactions and the absence of general signs [in rabbits], following vaccination with *Bacterium pneumosintes*, indicate that similar injections would be well tolerated in man."

In view of the specific protection afforded to rabbits and the absence of any defect in resistance to other infections as a result of subcutaneous vaccination with *Bacterium pneumosintes*, and having in mind the possible relationship of epidemic influenza to this microorganism, a group of fourteen volunteers at the Army Medical School in Washington, D. C., indicated their desire to submit to the vaccination. Nine of these persons had never had epidemic influenza. Four had suffered from the disease in 1918–19 and one in 1920.

These volunteers were accordingly given three graded doses of heat-killed cultures of *Bacterium pneumosintes*, and three of them were given a fourth and larger dose. Blood specimens were obtained before and after vaccination for comparison in agglutination tests. Leucocyte counts were also made before, and daily for 3 days after each injection, and the local and general reactions following each dose were noted. The small size of the group was compensated for by the opportunity to study the reactions of each person in detail.

Preparation of Vaccine.

Our observations indicated the cultural and antigenic identity of our strains of *B. pneumosintes*, whether derived from the epidemic waves of 1918–19, or 1920, or the recurrence of 1922. As in the instance of the vaccination carried

[4] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

out in the rabbit series, we again used two strains in preparing the vaccine; Strain 17, from the nasopharyngeal secretions of a patient attacked during the 1918–19 epidemic, and Strain 34, from similar secretions obtained during the 1922 recurrence of influenza. The organisms were grown in large quantities in *B. coli* broth,[5] prepared by adding 10 cc. of a 24 hour broth culture of *B. coli* to 1 liter of 1 per cent dextrose broth in a Florence flask and incubating for 1 to 2 hours at 37°C., or until the faintest visible haze of growth appeared in the clear medium. The medium was then heated in an Arnold steamer at 100°C. for 30 minutes under a petrolatum seal. The rapidly cooled broth, now sterile and anaerobic, was then seeded with 2 cc. of the respective cultures of *B. pneumosintes* grown in Smith-Noguchi medium. After 3 to 5 days incubation, heavy growths were obtained, which were centrifuged, washed with distilled water, and resuspended in salt solution as a stock vaccine. This was heated for 30 minutes at 55–56°C. and standardized by an opacity method.[6] It was then preserved with 0.35 per cent triple distilled cresol and before use it was diluted to correspond to a staphylococcus equivalent[3] of 2,000 million and 3,000 million organisms per cubic centimeter. After aerobic and anaerobic tests for viability and contamination proved negative, the vaccine was ready for use.

Dosage.

Of the fourteen volunteers (ten enlisted men and four officers) thirteen were studied as described below. The remaining man received only the first two injections and could not be further observed. A first dose of 0.5 cc. (1,000 million) and second and third doses of 1 cc. (2,000 million) were given. Three men received in addition a fourth dose of 1 cc. (3,000 million). All of the doses were estimated on the staphylococcus scale.

The interval between the first and second injections was 5 days (exceptions: 4 days in one instance and 8 in another) and between the second and third injections 8 days (exceptions: 7 days in three instances and 4 days in a fourth). In the three instances in which a fourth dose was given, the interval between the third and fourth doses was 10 days.

Reactions.

The first dose of 1,000 million was well tolerated. In only three instances were slight constitutional disturbances such as headache, mild depression, and generalized pains experienced. One of these

[5] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.

[6] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

men had slight chilly sensations also. The symptoms were transitory, beginning 8 to 10 hours after injection and lasting for a few hours only. All of the men had some tenderness and redness over an area 6 to 8 cm. in diameter at the site of injection, without any induration, as a rule. The local reaction subsided after 2 to 3 days. No one was even temporarily incapacitated.

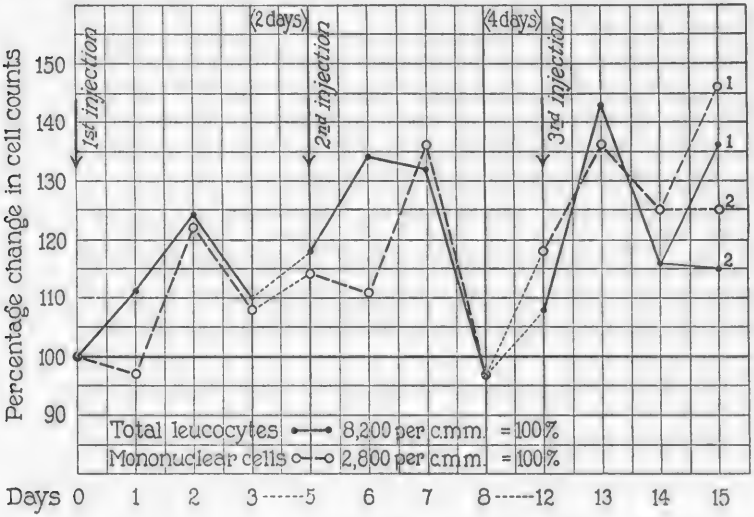
The second dose of 2,000 million was equally well borne. Again three men reported the general symptoms already mentioned. In no instance did the temperature rise above 99°F. (37.2°C.). Locally, the effects of the reaction were somewhat more severe, for induration was evident in areas 1 to 2 cm. in diameter, which persisted for 7 to 8 days. None of the volunteers was kept from their daily routine.

No constitutional reactions were experienced after the third dose of 2,000 million. The local reactions were similar to those following the second injection.

Finally, in view of the mildness of the reaction with the doses employed, three persons submitted to a fourth dose of 3,000 million, given 10 days after the third inoculation. The vaccine was injected at 10 a.m. At 8 p.m. on the same day one of the three showed a temperature of 99°F. (37.2°C.), but did not complain of any constitutional symptoms. The next morning the temperature was normal. However, there developed locally over an area 4 cm. in diameter some redness and induration attended with little or no tenderness. During the night the second man complained of headache, dryness of mouth and lips, and some weakness. The temperature was 99.4°F. (37.4°C.). By the next morning the temperature was normal and the constitutional symptoms had disappeared. The site of inoculation was tender and slightly indurated and the area of redness covered one-half of the posterior aspect of the upper arm. At 8.30 p.m. on the day of inoculation the third person showed a temperature of 100°F. (37.8°C.) and complained of dryness of the throat and slight pains in the muscles of the legs. By morning these symptoms had disappeared. Locally there was a red and tender area, without induration, measuring 5 to 6 cm. None of these three was incapacitated for duty by the injection. It may therefore be stated that the injection of doses of killed *Bacterium pneumosintes*, equivalent in amount to 3,000 million staphylococci, induces a milder reaction than that usually noted with the second or third dose of triple typhoid vaccine.

Effect on the Blood Count.

Leucocyte counts were made on nine of the vaccinated men before, and for 3 consecutive days after each injection. In each instance a rise in the total counts occurred, which carried the average for the nine men to 124, 134, and 143 per cent of its normal value, as shown in Text-fig. 1. In the chart the mononuclear cells have been plotted separately to show the differences in their behavior, especially after



TEXT-FIG. 1. The effect of subcutaneous injections of heat-killed suspensions of *B. pneumosintes* on the total and mononuclear leucocyte count.

the first and second injections, as compared with the total counts. It is interesting to note that, after the first and second injections, in spite of a rise in the total count which was part of the general reaction, the mononuclear cells suffered an absolute decrease. On the following day their recovery was disproportionately rapid and restored the normal ratio between them and the total count. The leucocytosis was transient and the totals fell again between the 2nd and 3rd days following injection.

At the time of the third injection the average count of the mononuclear cells was found to be high, and it rose to a new level during the following 24 hours, to fall again somewhat the next day. Two sets of final figures are given in the chart. On that day the counts of two of the men were recorded as 16,600 cells per c. mm., 4,000 cells more than any other count on that day and respectively 8,600 and 3,800 cells more than the corresponding figures for the day before. We have no explanation for these sudden and unexpected changes, for they were not accompanied by any observable clinical signs. With these two exceptions the counts throughout the series were fairly uniform, so that the average figures give a reliable picture of the general effects. If these exceptional figures are included, they shift the average counts to the points marked 1 on the chart. If excluded, the averages of the remaining counts fall at the points marked 2.

Production of Agglutinins.

The doses of vaccine given to the rabbits were relatively much larger than those administered to the men. They were followed by the appearance of specific agglutinins in the blood of the rabbits.[3] Agglutinins have also been demonstrated in the serum of seventeen of nineteen persons who had recently recovered from epidemic influenza.[7] In the present case of the vaccinated men tests for agglutinins were carried out by the method already described.[7]

An examination prior to vaccination of the blood serum of the men was made in dilutions of 1:2 to 1:10. No agglutinins against either of the strains of *Bacterium pneumosintes* used in the vaccine were found present. A second specimen was obtained from twelve of the vaccinated men on the 10th day, and from the other man on the 11th day after the third injection. The results of the agglutination tests with these sera are recorded in Table I.

An examination of the table shows that the blood of eleven of the thirteen vaccinated persons contained agglutinins against the two strains of *Bacterium pneumosintes* in serum dilutions of 1:4 to 1:40. In the serum of two, no agglutinins were detected. It is, of course, possible that agglutinins might have been found in the blood of these

[7] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1923, xxxvii, 303.

men had more specimens been studied. Such variations in antibody response are, of course, known for other organisms.[8,9]

In view of the mildness of the reactions in this preliminary series, and the harmlessness of the injections, Lieutenant-Colonel Craig has offered the *pneumosintes* vaccine to a much larger group of men at

TABLE I.

Agglutination of Serum after Vaccination.

Case No.	Strain 17.*				Serum + buffer.	Strain 34.*			
	1:4	1:10	1:20	1:40	1:4	1:4	1:10	1:20	1:40
1	+++	++	−	−	−	+++	++	+	−
2	+++	++	−	−	−	+++	++	+	−
3	+++	++	−	−	−	+++	+	−	−
4	+++	++	−	−	−	++	−	−	−
5	−	−	−	−	−	−	−	−	−
6	++	−	−	−	−	++	+	−	−
7†	++	−	−	−	−	+	−	−	−
8	+++	++	+	−	−	+++	++	++	+
9	++	−	−	−	−	+	−	−	−
10	+	+	−	−	−	++	+	−	−
11	−	−	−	−	−	−	−	−	−
12	+	−	−	−	−	+	−	−	−
13	++	+	−	−	−	+	−	−	−
No serum.	−	−	−	−	0	−	−	−	−

* Strain 17 was obtained from the 1918–19 wave, and Strain 34 from the 1922 wave of the influenza epidemic.

† The specimen from this person was obtained 11 days after the last injection. Specimens from the remainder were collected 10 days after the vaccination series.

+++ indicates complete agglutination with clear supernatant fluid; ++, almost complete agglutination with slightly clouded supernatant fluid; +, distinct agglutination with clouded supernatant fluid; −, no agglutination or serum flocculation; and 0, no test made.

Fort Meyer, who have availed themselves of the opportunity. Should there be a recurrence of epidemic influenza in the near future, the efficacy of the vaccination may receive a practical test.

[8] Maverick, A., *J. Am. Med. Assn.*, 1912, lviii, 1672. Trowbridge, E. H., Finkle, B. A., and Barnard, E. M., *J. Am. Med. Assn.*, 1915, lxiv, 728.

[9] Gates, F. L., *J. Exp. Med.*, 1918, xxviii, 449.

SUMMARY.

Thirteen volunteers were injected subcutaneously with a vaccine prepared from two strains of *Bacterium pneumosintes*. Three doses were given corresponding, by opacity tests, to 1,000, 2,000, and 2,000 million staphylococci. The intervals between the several injections were 5 and 8 days. Local reactions of mild and transitory character only were noted. Constitutional reactions, also mild and transitory, consisting of headache, depression, and generalized muscular pains, were infrequently observed. None of the men was prevented by the vaccine from pursuing his ordinary duties. The vaccinations induce a transitory leucocytosis and lead to the appearance of specific agglutinins in the blood serum.

STUDIES ON THE RELATION OF TETANUS BACILLI IN THE DIGESTIVE TRACT TO TETANUS ANTITOXIN IN THE BLOOD.

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(Received for publication, October 30, 1922.)

In a previous paper (1) we have reported the isolation of *Bacillus tetani* from 34 per cent of the stools of 78 individuals in Peking, and we have discussed the relation of this high carrier incidence to the number of infections with tetanus bacilli. It is difficult to draw any conclusions regarding this relation as it is impossible to get accurate statistics, but we have been impressed by the statement of a number of foreign physicians practising in North China who agree in saying that they have seen very few cases of tetanus. In order to explain why infections with this organism are relatively uncommon in a region where a third of the population are eliminating tetanus bacilli in their stools and where human feces are so generally distributed, we asked ourselves the question: Do tetanus bacilli in the digestive tract cause the production of antibodies, and in particular antitoxins, which protect the host against infections with these organisms? In this paper we wish to report on the presence of tetanus antitoxin in the serum of individuals who carry tetanus bacilli in their digestive tracts.

When one considers the importance of this subject the records of the examination of the blood of man and animals for tetanus antitoxin are remarkably scarce. We have been able to find only the following, though there may be incidental observations buried in the extensive literature on tetanus in general. Hamburger (2) says that he has never found a trace of tetanus antitoxin in the blood of normal animals or man. Since he does not give any experimental evidence to support his statement, we do not know how many specimens he examined or what methods he used, but it is worthy of note that this is the only statement regarding the examination of human blood that we have found. Although Buxton and Glenny (3) failed to find tetanus antitoxin in the blood of 500 horses, we know from the observations of Sanchez-Toledo and Veillon (4), Choukévitch (5), Lukas (6), and

Noble (7) that these animals not infrequently carry tetanus bacilli in their digestive tracts. However, Römer (8) has examined the blood of normal cattle for antitoxin and found it present in 13 of the 39 animals studied. Of 17 cattle under 2 years of age only 1 showed antitoxin and this was a calf aged 1½ months whose mother had a high antitoxin content in her blood. Of the 22 animals over 2 years old, 12 showed antitoxin and, in some cases, large amounts of it. Römer examined the stools of only 1 of the cows having antitoxin and found tetanus bacilli present, but since others had shown that a large percentage of cattle carried tetanus bacilli in their digestive tracts and since he found antitoxin only in the blood of the older cattle, he assumes that this antitoxin is due to the presence of the bacilli in the digestive tract. We feel that his explanation is probably correct but that it would be more convincing had he shown that the cattle having antitoxin in their blood carried tetanus bacilli, while those having no antitoxin did not carry these organisms.

Method Used in Testing Sera for Antitoxin.

In all of our tests for antitoxin we have used portions of a single lot of dried tetanus toxin, prepared by the method of Brieger and Fraenkel (9), that was kept in a vacuum over phosphorus pentoxide. The M.L.D. of this toxin has remained remarkably constant at about 0.01 mg. throughout our work. For test animals we have used field mice as they are larger and more easily obtained than white mice. Per gram of body weight the former are more susceptible than the latter, as shown in the results of one of our experiments given in Table I, and it will also be seen that the M.L.D. for a 12 gm. white mouse is about the same as that for a 25 gm. field mouse.

For each day's test 0.1 gm. of toxin was carefully weighed and suspended in sterile 50 per cent glycerol, and dilutions from this suspension were mixed with equal quantities of the serum to be tested which was diluted 1:5. After incubation at 37°C. for 30 minutes, each mixture was injected into two field mice, each mouse receiving 1 cc. subcutaneously near the root of the tail. A complete protocol of the examination of one serum is given in Table II. Since the field mice varied considerably in weight we made it a practise to use on a single day those that weighed within 5 gm. of one another. Control mice of the same weight were always inoculated. As a rule, three specimens of sera from individuals who carried tetanus bacilli and three from individuals who did not were tested on the same day. After 4 days the final record on the animals was made, and those surviving were not used subsequently.

TABLE I.

Test to Compare the Resistance, to Tetanus Toxin, of Field and White Mice. March 15, 1922.

Field mice.			White mice.		
Weight of mouse.	Toxin injected.	Result.	Weight of mouse.	Toxin injected.	Result.
gm.	*mg.*		*gm.*	*mg.*	
21.5	0.005	Dead in 4 days.	12	0.005	Dead in $3\frac{1}{2}$ days.
21.5	0.005	" " $3\frac{1}{2}$ "	14.5	0.005	Generalized tetanus; survived.
20	0.0075	" " $3\frac{1}{2}$ "	13	0.0075	Dead in $3\frac{1}{2}$ days.
21	0.0075	" " 66 hrs.	12	0.0075	" " 48 hrs.
20.5	0.01	" " 40 "	12	0.01	" " 66 "
22.5	0.01	" " 48 "	11.5	0.01	" " 48 "

TABLE II.

Protocol of the Examination, for Antitoxin, of the Serum of a Normal Woman Who Showed Tetanus-Like Bacilli in Her Feces.

Weight of mouse.	Amount of toxin injected.	Amount of serum injected.	Results.
gm.	*mg.*	*cc.*	
21	0.02	0.1	No effect.
21	0.02	0.1	" "
23	0.05	0.1	" "
21	0.05	0.1	" "
22	0.1	0.1	" "
21	0.1	0.1	" "
22	0.25	0.1	" "
21	0.25	0.1	" "
22	0.5	0.1	Generalized tetanus; dead in 66 hrs.
22	0.5		
20	0.01	Control.	Slight spasms 40 hrs.; dead in $3\frac{1}{2}$ days.
23	0.01	"	" " 40 " " " 4 "
23	0.02	"	Spasms 40 hrs.; dead in 66 hrs.
20	0.02	"	" 40 " " " 66 "
25	0.03	"	" 40 " " " 48 "
24	0.03	"	" 40 " " " 48 "

Results Obtained from the Examination of 56 Individuals.

Our first tests were made on the sera of individuals from whose stools we had either isolated tetanus bacilli or had failed to find these

TABLE III.

Summary of the First Series of Examinations Showing the Relation of Tetanus Spores in the Feces to Antitoxin in the Blood.

Patient No.	*B. tetani* in stool.	Effect of injection of 0.1 cc. of serum mixed with tetanus toxin as indicated.						
		Date of test.	Toxin injected.		Mouse A.		Mouse B.	
					Weight.	Result.	Weight.	Result.
		1922	*mg.*	M.L.D.	*gm.*		*gm.*	
340	Isolated.	Jan. 10	0.05	5	28	No effect.	21	No effect.
			0.1	10	21	t; recovered.	21	t; recovered.
			0.25	25	20	ttt + 3½ days.	25	ttt + 3½ days.
374	"	" 10	0.1	10	20	No effect.	18	t; recovered.
			0.25	25	26	ttt + 3½ days.	20	ttt + 3½ days.
451	Absent.	" 10	0.02	2	29	ttt + 4 "	38	ttt + 66 hrs.
Controls.			0.005	½	24	t; recovered.	36	t; recovered.
			0.01	1	27	ttt + 5 days.	26	ttt + 3½ days.
			0.02	2	25	ttt + 40 hrs.	26	ttt + 72 hrs.
317	Isolated.	Jan. 11	0.1	10	25	No effect.	3	No effect.
			0.25	25	21	ttt + 66 hrs.	29	t; recovered.
932	"	" 11	0.25	25	25	No effect.	21	No effect.
			0.5	50	26	t; recovered.	28	t; recovered.
172	"	" 11	0.05	5	35	No effect.	28	No effect.
			0.1	10	25	t; recovered.	22	t; recovered.
			0.25	25	26	ttt + 66 hrs.	32	ttt + 66 hrs.

138	Absent.	Jan. 11	0.02	2	31	ttt + 66 hrs.	22	ttt + 3½ days.
288	"	" 11	0.02	2	34	ttt + 3½ days.	25	ttt + 3½ "
439	"	" 11	0.02	2	42	ttt + 3½ "	22	ttt + 66 hrs.
Controls.		" 11	0.005	½	26	t; recovered.	25	t; recovered.
			0.01	1	27	tt; recovered.	26	ttt + 4 days.
			0.02	2	31	ttt + 42 hrs.	24	ttt + 66 hrs.
152	Isolated.	Jan. 17	0.1	10	25	No effect.	25	+ 66 hrs. Not in tetanic condition.
			0.25	25	26	ttt + 40 hrs.	27	ttt 40 hrs.
391	"	" 17	0.25	25	26	No effect.	25	No effect.
			0.5	50	25	ttt + 40 hrs.	25	ttt + 40 hrs.
228	Absent.	" 17	0.02	2	25	tt; recovered.	27	ttt + 4½ days.
			0.05	5	28	ttt + 66 hrs.	27	ttt + 40 hrs.
393	"	" 17	0.02	2	28	ttt + 3 days.	29	ttt + 4 days.
394	"	" 17	0.02	2	28	ttt + 4 "	27	ttt + 3½ "
Controls.		" 17	0.01	1	28	ttt + 4 "	27	ttt + 3 "

t indicates slight spasm of one leg; tt, moderate tetanus; ttt, generalized tetanus.

organisms. We have summarized these tests in Table III, giving only the largest amount of toxin that was neutralized by each and the smallest amount that was not neutralized. Although all of the specimens of sera were tested against the amounts of toxin given in Table II, the complete protocols are not given because of their length and the fact that they would add little to the value of the report. Our results show that in not a single instance did 0.1 cc. of serum from seven individuals whose stools were free from tetanus bacilli neutralize 2 M.L.D., whereas the same amount of serum from the seven individuals whose stools contained tetanus bacilli neutralized 10 M.L.D. of toxin, and in one case (No. 932) 0.1 cc. practically neutralized 50 M.L.D. of the toxin.

TABLE IV.

Summary of the Tests Made on the Sera of 42 Individuals, Showing the Relation of Tetanus-Like Bacilli in the Feces to Antitoxin in the Blood.

Result of stool examination.	No. of sera with the maximum amount of tetanus toxin neutralized by 0.1 cc.					
	2 M.L.D. not neutralized.	2 M.L.D.	5 M.L.D.	10 M.L.D.	25 M.L.D.	50 M.L.D.
Tetanus-like bacilli present.	0	1	1	4	10	3
" " absent.	21	0	0	2	0	0

In our previous work we had shown that we could almost invariably isolate tetanus bacilli whenever the stained film of the sediment from a fermentation tube inoculated with the heated suspension of the feces showed typical tetanus-like organisms. Since this isolation of tetanus bacilli from mixtures containing great numbers of other anaerobes is a difficult and time-consuming process, in our next series of tests we determined the presence or absence of tetanus-like bacilli in the feces and did not attempt to isolate them. The sera of nineteen individuals whose stools contained tetanus-like organisms and the sera of twenty-three whose stools were negative were tested for antitoxin as in the preceding series. The results of these tests are summarized in Table IV, and they show that in all of the cases where tetanus-like organisms were found in the feces, there was a

definite neutralization of the toxin by the serum. On the other hand, the toxin was neutralized by the sera from only two of the twenty-three individuals whose stools were apparently free from tetanus bacilli.

If we combine these two series, we find that all of the sera from the twenty-six individuals whose feces showed tetanus-like bacilli contained antitoxin, while of the thirty whose stools were free from these organisms, only two did so; 0.1 cc. of the sera of these two neutralized 10 M.L.D., while the same amount of the remaining twenty-eight failed to neutralize 2 M.L.D. of toxin.

Percentage of Individuals Having Antitoxin in Their Serum.

As the relation of antitoxin in the serum to tetanus bacilli in the intestinal tract is so constant we have a means of checking our previous work on the percentage of individuals who act as carriers of this organism. We secured from the Wassermann laboratory forty specimens of serum, most of them from normal individuals, such as employees of the institution and soldiers from one of the legations. From each specimen two mice were inoculated subcutaneously, each mouse receiving a mixture that contained 0.1 cc. of serum and 10 M.L.D. of toxin. Of the forty specimens tested, fifteen, or 37.5 per cent, neutralized the 10 M.L.D. of toxin, a figure that agrees very closely with the results of our stool examination which showed the presence of tetanus bacilli in 34 per cent of the individuals examined.

Estimated Antitoxin Content of Serum of Carriers of Tetanus Bacilli.

We have been unable to compare our toxin with accurately standardized antitoxin, but we have tested it against two lots of antitoxin put up for therapeutic purposes. One lot from the Bureau of Science, Manila, where the United States Army unit is used, required 0.1 unit to neutralize 100 M.L.D of our toxin. The other lot from the Kitasato Institute for Infectious Diseases, where the German unit is used, required 0.002 unit to neutralize 100 M.L.D. of the same toxin. These figures agree well with MacConkey's (10) statement that the German unit equals 40 United States Army units, but we feel that they are only approximate as the strength of antitoxin

may vary greatly from the figure on the container. We can say, however, that individuals who carry tetanus bacilli in their digestive tracts have an appreciable amount of antitoxin in their blood since 0.1 cc. of most of our sera neutralized 10 and, in many cases, 25 M.L.D. of toxin. We hope that someone who has access to accurately standardized toxin will study the blood of carriers of tetanus bacilli and determine more accurately the amount of antitoxin present.

DISCUSSION.

Our question as to whether the presence of tetanus bacilli in the digestive tract leads to the production of antibodies by the host is thus answered in the affirmative, and we can understand why in a region where a third of the population carry these bacilli, infections are relatively uncommon. We can also understand why we find so few cases of tetanus following dysentery, typhoid, and operations on the intestine.

A carrier of tetanus bacilli is potentially a disseminator of these organisms, but he is not so great a menace to his fellow men as the horse, because his feces are as a rule disposed of more promptly. On the other hand, it is to his advantage to become a carrier for he thereby acquires an immunity, which, while it may not be absolute, may be of great service to him should tetanus bacilli enter his body through a wound.

If we could be sure that ingested spores would, in every case, grow in the intestines, we should have an easy method of immunizing a large body of men to tetanus bacilli. Tulloch's findings (11) indicate that many of the men who were in France during the recent war became carriers of tetanus bacilli, and it is probable that many lives were thereby saved. Would it have been wise to have made all of these men carriers so that they could have produced their own immune bodies? From what we see in China, we might think so, but we must not forget that such a procedure would result in a wide distribution of tetanus bacilli, the evil effects of which might counterbalance the good effects of the immunization. The immunity resulting from the ingestion of spores is probably only relative, and if bacilli in great numbers were introduced into wounds from badly contaminated soil it is probable that the body would be overwhelmed.

It is probable, therefore, that a better procedure, in case of another great war, would be to immunize men by the injection of toxin-antitoxin mixtures as used by Buxton and Glenny (3) for protecting horses against tetanus.

Our attempt to study the production o fantitoxin following the establishment of tetanus bacilli in the human digestive tract has as yet yielded no satisfactory results. For this experiment one of us swallowed a large number of mature spores. A period of marked constipation followed, which may or may not have been due to the presence of tetanus bacilli in the intestines. Before the spores were ingested no tetanus bacilli were present in the feces, while for the first 10 days after they were ingested, they were present in great numbers. At this time it became necessary to take a drastic cathartic on account of the constipation, and afterwards no more spores were found in the feces. We were more successful in a monkey and later we will report the studies made on the blood of this animal after the establishment of tetanus bacilli in its digestive tract.

It seems possible that the explanation of the varying susceptibility of guinea pigs to tetanus toxin as noted by Taber (12) is due to the fact that these animals may carry tetanus bacilli in their digestive tracts (7). Whether these carriers develop antitoxin is a question we have not investigated, but it seems probable that they behave just as men do in this respect.

Whether the antitoxin found in the blood of carriers of tetanus bacilli is due to absorbed toxin or to the occasional entrance of the organisms into the body is a question we cannot answer. If, however, the latter were the case, we should expect cases of idiopathic tetanus to be relatively common; yet so far we have been unable to get a single record of idiopathic tetanus in this region.

From the clinical standpoint, a quick method of testing for antitoxin in the blood is desirable. The agglutination test cannot be used for we find that sera free from antitoxin will often agglutinate the type of tetanus bacillus that predominates here in as high dilutions as does serum containing antitoxin. Neutralization tests are not practical as a common laboratory procedure for the toxin must be freshly made up for each test, its strength must be determined, and the results of the test are not known for some days. However,

should it be desirable to test a large body of men to determine their immunity, the injection of 0.1 cc. of serum and 10 M.L.D. of toxin into mice is practical and is a comparatively simple procedure.

The finding of agglutinins of tetanus bacilli in sera free from antitoxin may help us in answering a question that has puzzled us for some time. As we have stated above, tetanus infections are relatively infrequent here, yet our results show that only approximately a third of the population have tetanus antitoxin in their blood. The remainder of the population certainly have had numerous opportunities to ingest the bacillus and the bacilli have either been unable to grow because of antagonistic organisms, or they have grown and been cast off. The finding of agglutinins in the blood of a certain percentage of individuals whose serum contains no antitoxin and whose stools are free from the bacillus points to the fact that the latter explanation is the correct one. If this is the case, we must assume that the agglutinins have persisted longer than the antitoxins, and then the question arises as to whether these people really are immune. We should expect their bodies to be trained in antitoxin production, and in case of an infection with tetanus bacilli to respond more quickly than the body of an individual who had never had tetanus bacilli in his digestive tract or produced antitoxin. Glenny and his associates (13, 14) have shown that this quick response occurs in animals that have previously been immunized with diphtheria toxin. At the time of the second injection of toxin their blood may be free from antitoxin, but they produce it much more rapidly than a normal animal. The assumption is that they are actively immune. We therefore feel that the relatively low incidence of tetanus in North China is due, in part, to the fact that the majority of the population have a potential immunity because they carry or have carried tetanus bacilli in their digestive tracts. Other factors may be the intense sunlight, which may sterilize the soil, and the few industrial accidents.

CONCLUSIONS.

1. The sera of twenty-six individuals who carried tetanus bacilli in their digestive tracts all contained appreciable amounts of antitoxin.

2. The sera of thirty individuals in whose stools no tetanus-like organisms were found were, with two exceptions, free from tetanus antitoxin.

3. Although we have been unable to measure accurately the antitoxin content of these human carriers of tetanus bacilli, 0.1 cc. of serum neutralizes 10 or more M.L.D. of toxin and it is evident that they have acquired an active immunity due to the bacilli in the intestinal tract.

4. These results definitely prove that tetanus bacilli grow in the intestinal tract of man.

5. Many of the individuals who have no tetanus bacilli in their intestinal tracts and whose serum is free from antitoxin show agglutinins to tetanus bacilli. It is probable that they have been carriers of the bacilli in the past and that the agglutinins have persisted longer than the antitoxins. It seems likely, therefore, that these individuals are potentially immune to tetanus.

6. If tetanus bacilli can be established in the digestive tract of man we have a means of immunization which might be useful in armies or in regions where tetanus infections are common, though we do not recommend this method of immunization at present.

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THE RELATION OF ANTIBODY TO THE RATE OF DISAPPEARANCE OF CIRCULATING ANTIGEN.

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(Received for publication, October 19, 1922.)

In a previous paper (1) with Leake we reported a study of the relation of circulating antibody and antigen to susceptibility to serum disease in a group of patients to whom antipneumococcus or antimeningococcus serum had been administered therapeutically. This work was suggested by the observations which Longcope and Rackemann (2) had reported. The results seemed to justify the conclusion that individuals who have severe serum disease are good precipitin formers, and that at the time the precipitin in the circulation reaches the crest of its curve or soon afterwards the precipitinogen (antipneumococcus or antimeningococcus serum from immunized horses) rapidly disappears from the blood stream. On the other hand, in those individuals who, after a first administration of foreign serum, show very mild or no symptoms of serum disease little or no precipitin is demonstrable in the patient's serum, and the precipitinogen persists in the circulation for a long period. Intermediate types were also encountered. Since the rapid disappearance of the foreign serum appeared to be dependent upon the presence of circulating precipitin in high titer, one might assume that the precipitin disposed of the foreign serum by uniting with it in the circulation. However, it was suggested that another mechanism might serve equally well to rid the circulation of the foreign serum without assuming that an intravascular union played an important part in the mechanism of disposal. At the period when precipitin is abundant both within the tissue cells and in the circulation, one might plausibly assume that with greater amounts of intracellular antibody available the tissue cells would appropriate the antigen with greater avidity.

Since, in the presence of circulating antibody, there is no way of measuring the avidity of tissue cells for antigen brought to them in the circulation we can arrive at a conclusion regarding the mechanism of disappearance of antigen only by excluding the other possibility or proving its effectiveness. The experiments reported in the present paper were designed to exclude or establish intravascular union as the mechanism of disposal, and also to determine whether or not the relations of antibody and antigen in the circulation of animals give curves similar to those obtained in man with the same antigen. Incidentally, during the course of the experiments we have noted some phenomena bearing on the behavior of antigen and antibody in the circulation for which satisfactory explanation is lacking.

The literature contains several studies (2–7) bearing on the problem under consideration, but in the previously reported work the effort has been to determine how much sensitized animals differ from normal animals in the rate of disappearance of antigen. A review of this literature is unnecessary. It may be summarized by stating that the evidence is in favor of a more rapid disappearance of antigen from the circulation of sensitized or immunized animals as compared with normal controls. None of these investigators, however, considered the titer of antibody in the circulation at the time of reinjection, the experiments for the most part having been carried out on animals sensitized by a single preparatory injection. If it be the precipitin of sensitized animals which disposes of the specific antigen a more rapid disappearance would be expected in highly immunized animals with an abundance of precipitin in the circulation than in animals sensitized by a single injection which appear to dispose of antigen at a moderately increased rate compared to normal animals.

Except for Hempl (7), those who have worked at this problem have not given, we believe, sufficient consideration to the great individual variation of experimental animals in their ability to form antibody and to rid the circulation of antigen.

Method.

The determinations of antigen in the circulation were made by specific precipitation with the serum of rabbits immunized against horse serum.

The rabbits furnishing this precipitating serum were prepared by three injections (0.5, 1, and 1.5 cc.) of horse serum at intervals of 4 days. 5 to 8 days after the last injection a titration was done and the serum tested for persisting traces of antigen. If the titer was 1:20,000 or higher and no antigen was demon-

strable by setting the serum up in dilutions against another anti-horse rabbit serum, previously found to be free from antigen, the rabbit was bled to death from the carotid artery and the serum separated and diluted with an equal volume of normal salt solution. None of the precipitating serums was inactivated and no preservatives were used. The circulating precipitin was determined by setting up the serum being tested, diluted with an equal volume of normal salt solution, against dilutions of normal horse serum. In all the tests 0.5 cc. of the serum containing, or being tested for antibody, was added to 0.5 cc. of the serum containing, or being tested for antigen. Controls with salt solution and with dilutions of normal rabbit serum and normal sheep serum were set up each day that tests were done. The tubes were placed in a water bath at 37.5°C. for 1 hour, then in the ice box over night, when the final reading was made.

The critical injection of antigen was given, both in immunized and unimmunized animals, in fractional doses over a period of about 90 minutes.

Horse serum was used as antigen because the study of the relation of antibody to the disappearance of antigen in patients with serum disease was of necessity a study of the fate of injected horse serum and the relations within the circulation of the horse serum and its specific precipitin. Another advantage in using serum is the fact that it is not excreted by the kidneys. After intravenously injecting horse serum both in patients and in laboratory animals we have repeatedly tried to demonstrate it in the urine by specific precipitation. Without exception these attempts have been unsuccessful. With egg albumin and many antigens of bacterial origin this is not the case. Oppenheimer (8) found that as much as 49 per cent of intravenously injected egg albumin may be excreted by the kidneys. Zinsser (9) has also noted the renal excretion of egg albumin, and we have confirmed this observation. In our experiments the amount of horse serum injected was approximately the equivalent for a rabbit, on the basis of body weight, of the amount administered therapeutically to the subjects in our study of serum disease.

RESULTS.

It would require too much space to protocol all the earlier experiments. They comprise observations on twenty-three rabbits. Twelve of these were immunized before the test injection of antigen; eleven were controls to which, without prior immunization, antigen was administered in the same way and in amounts equal, in each experi-

ment, to the amounts administered to the previously immunized animals. A summary of the results of these preliminary observations is given in Tables I and II. The data of these tables make it possible to study the relation of circulating antibody to the rate of disappearance of circulating antigen under three different sets of conditions.

TABLE I.

Experiment No.	Rabbit No.	Amount of antigen injected (intravenously).	Persistence of antigen.
		cc.	*days*
1	1	3.00	14
	2	3.00	17
	3	3.00	17
	4	3.00	14
2	5	6.00	8
	6	6.00	6
3	7	6.00	21
	8	6.00	21
	9	6.00	21
	10	6.00	21
5	11	6.00	14

Rabbits 1 to 11 were controls without preparatory immunization.

One is struck by the approximate uniformity of behavior of the rabbits in each experiment and the wide limits of variation in the rate of disappearance of antigen in different experiments. That this was not due to a difference in the precipitating power of the anti-horse rabbit serum used to demonstrate the circulating antigen is evident from the fact that the antigen determinations were frequently checked by using more than one anti-horse rabbit serum for the animals in the same experiment and by using the same anti-horse rabbit serum in more than one experiment. Whether the uniformity in each experiment and the variation in persistence of antigen in different experiments can be due to differences in the horse serum used as antigen is not clear. All the horse serum used was obtained from the Department of Health of the City of New York. All the animals in each experiment received serum from the same lot, but a different lot of serum may have been used in each experiment. It seems more probable that there is a wide variation in the rate with which individual animals dispose of foreign serum in the circulation, and that animals of the same litter tend toward uniformity. The animals used in each experiment were received from the breeder at the same time but whether they belonged to the same litter or not is uncertain.

TABLE II.

Experiment No.	Rabbit No.	Amount of antigen injected.	Persistence of antigen.	Precipitin in circulation at time of reinjection.
		cc.	*days*	
1	12	3.00	6	None.
	13	3.00	8	Moderate.
	14	3.00	17	"
	15	3.00	14	Strong.
2	16	6.00	29	"
	17	6.00	37	Weak.
3	18	6.00	0	Strong.
	19	6.00	3	"
	20	6.00	1	"
	21	6.00	3	"
4	16*	6.00	29	None.
	5*	6.00	8	"

* Rabbit 16 appears twice in this table because, as already mentioned, it was first used in an experiment to determine the rate of disappearance of antigen in an animal with circulating antibody and then later used again in an experiment to determine the rate of disappearance in an animal previously immunized but containing no free circulating antibody at the time of reinjection. For a similar reason Rabbit 5 appears in Tables I and II.

1. Comparison of the Rate of Disappearance of Circulating Antigen in Immunized Animals with a High Titer of Antibody at the Time of Reinjection with the Rate of Disappearance in Animals Which, Following Immunization, Develop Little or No Precipitin.—Table III records the results in one animal of each of these types. Rabbit 12 was a poor antibody former and had no circulating precipitin at the time that 3 cc. of horse serum were injected intravenously. Rabbit 15, on the contrary, was a good precipitin former and had a high titer of precipitin at the time of reinjection. Both animals had been given the same preparatory immunization. It is seen that the antigen disappeared somewhat more rapidly from the circulation of Rabbit 15 during the first 3 days but that complete disappearance occurred at the same time in the two rabbits. In other experiments the rate of

TABLE III.

Antigen or antibody.	Final dilutions.	Before reinjection.	No. of days after reinjection (3 cc. of horse serum intravenously).													
			1	3	6	8	11	14	17	21	29	36	43	50	57	64
Rabbit 12, weight 1,900 gm.*																
Precipitinogen.	1:20,000	0	0	0	0	0	0	0	0	0	0					
	1:2,000	0	0	0	0	0	0	0	0	0	0					
	1:200	0	+	+	0	0	0	0	0	0	0					
	1:20	0	+++	++	+	0	0	±	0	0	0					
	1:2	0	+++	+++	++	0	0	0	0	0	0					
Precipitin.	1:200,000	0	0	0	0	0	0	0	0	0	0					
	1:20,000	0	0	0	0	0	0	±	0	0	0					
	1:2,000	0	0	0	+	±	±	+	±	0	0					
	1:200	0	0	0	+	0	±	±	0	0	0					
	1:20	0	0	0	0	0	0	0	0	0	0					
	1:2	0	0	0	0	0	0	0	0	0	0					

Rabbit 15, weight 1,900 gm.*

Precipitinogen.	1:20,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1:2,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	1:200	0	+	0	0	0	0	0	0	0	0	0	0	0	0	
	1:20	0	+	+	+	0	0	±	0	0	0	0	0	0	0	
	1:2	0	++	++	++	0	0	+	0	0	0	0	0	0	0	
Precipitin.	1:200,000	+	0	+	0	+	+	+	+	+	±	+	±	±	+	±
	1:20,000	+	0	+	+	+(+)	+(+)	++(+)	++(+)	+(+)	+	+	+	+	+	+
	1:2,000	++	0	+	+++	+++	+++	+++	+++	++	+	+(+)	+	+(+)	+	+
	1:200	+++	+	++	++++	++++	++++	++++	++(+)	++(+)	+(+)	++	++	+	+	+
	1:20	+++	+	+++	++++	++++	++++	++++	++++	++	±	+	+	±	±	0
	1:2	±	±	±	+	++(+)	+(+)	±	+(+)	0	0	0	0	0	0	0

In Tables III to VIII the results of precipitation are recorded according to the following scheme: ± cloud, but no precipitate; + definite precipitate; +(+) slightly heavier precipitate than +; ++, ++(+), +++, and +++(+) increasing amounts of precipitate; and ++++ heavy precipitate occupying about one-fourth to one-third of the column of fluid in the tube.

*Rabbits 12 and 15 were prepared by three intravenous injections of 0.5, 1, and 1.5 cc. of horse serum at intervals of 4 days. The last of the immunizing injections was given 12 days before the reinjection with 3 cc. of horse serum. The two animals showed complete disappearance of antigen on the same day although the one (Rabbit 15) with circulating antibody at the time of reinjection showed a somewhat more rapid diminution of circulating antigen during the first 3 days.

disappearance in animals with an abundance of circulating antibody was much more rapid than in animals with little or no antibody, but there were also observations in which the animal poor in antibody disposed of circulating antigen more rapidly than the animal rich in antibody. In short, the association of rapid disappearance of antigen with a high titer of antibody, although several times observed, was far from constant.

2. Comparison of the Rate of Disappearance of Antigen in Unimmunized and Immunized Animals.—The average time that antigen was demonstrable in the circulation of the four unimmunized animals which received 3 cc. of horse serum was 15.5 days; in the previously immunized animals receiving 3 cc. the antigen was demonstrable for an average of 11.2 days. In the rabbits receiving 6 cc. of horse serum the average figures were: unimmunized 16.3 days, immunized 13.7 days. Certainly the difference, while in favor of the immunized animals, falls short of theoretical expectations. Table IV shows the results on one unimmunized animal and one previously immunized. Despite the fact that the latter had a high titer of circulating antibody at the time of reinjection it retained the antigen in the circulation longer than the unimmunized control. However, other instances in which the reverse occurred were also observed.

3. Comparison of the Period of Persistence of Injected Antigen in the Same Animal on Two Occasions: (a) When It Has Free Antibody in the Circulation, and (b) Later When It Has None.—Rabbit 16 affords an instance of this. In September, 1920, it was immunized and then reinjected with 6 cc. of horse serum at a time when there was a high titer of circulating precipitin. The result of this experiment is shown in Table IV. It was then kept in a cage without interference of any kind until July, 1921. At this time the serum gave no reaction for either precipitin or precipitinogen. On July 11, it was again injected with 6 cc. of horse serum. The subsequent relations of precipitin and precipitinogen are shown in Table V. It is of some significance that on the second occasion, despite the absence of circulating precipitin, antigen disappeared more rapidly than on the first occasion when 6 cc. of horse serum were injected.

This group of preliminary experiments may be summarized by the statement that the results failed to give a clear indication that cir-

culating antibody plays an important part in disposing of circulating antigen. The rabbits previously immunized did, to be sure, tend to rid the circulation of antigen somewhat more rapidly than unimmunized controls, but the difference was not impressive and there were significant exceptions. Furthermore, among the immunized animals the rate of disappearance bore no constant relation to the titer of circulating antibody, and it was found that the same animal may dispose of circulating antigen as rapidly at a time when there is no circulating antibody as when there is a high titer.

In addition to the observations on the relation of circulating antibody to the rate of disappearance of circulating antigen, these experiments bring out several other phenomena concerning the existence and behavior of antigen and antibody in the blood stream.

1. Coexistence of Ununited Antibody and Antigen in the Circulation.—This phenomenon has stimulated a number of experimental efforts to determine whether the apparent coexistence of an antigen with its specific antibody is in fact what it seems to be. The results are not in complete agreement. While our experiments were not designed to elucidate this problem it is interesting to note the frequency and duration of the apparent coexistence of precipitinogen and specific precipitin. The more important studies of this question may be found in the papers of Uhlenhuth and Weidanz (10), von Dungern (11), Torikata (12), Zinsser and Young (13), Weil (14), Denzer (15), and Bayne-Jones (16).

2. Reappearance of Antigen after It Had Ceased to Be Demonstrable in the Circulation.—This phenomenon we first noted during the course of the study of the relations of antigen and antibody in serum disease (1). In several of those patients the precipitinogen, after disappearing from the circulation, reappeared again following an interval of several days, during which none could be demonstrated in the serum. This same reappearance is recorded five times in the series of twenty-three rabbits. In connection with this observation it is interesting to note that Vaughan, Cumming, and McGlumphy (17) found that egg white which had passed from the circulation into the tissues could be "washed" back into the circulation by infusions of salt solution.

TABLE IV.

Antigen or antibody.	Final dilutions.	Before reinjection.	No. of days after reinjection (6 cc. of horse serum intravenously).										
			1	3	6	8	10	13	17	22	29	38	45
			Rabbit 16, weight 2,200 gm.										
Precipitinogen.	1:20,000	0	0	0	0	0	0	0	0	0	0	0	0
	1:2,000	0	+	0	0	0	0	0	0	0	0	0	0
	1:200	0	+	0	0	0	0	0	0	0	0	0	0
	1:20	0	++	+	+	0	0	0	0	0	0	0	0
	1:2	0	+(+)	±	++	+	+(+)	+	±	0	+	0	0
Precipitin.	1:2,000,000	0	0	0	+	+	+	+	±	+	0	0	0
	1:200,000	+	0	+	++	++	++	++	++	+	0	0	0
	1:20,000	+	0	+	++(+)	+++	++	++(+)	++(+)	++	+	+	±
	1:2,000	++(+)	0	+	+++(+)	+++(+)	+++	+++	+++	++(+)	+	+	±
	1:200	++(+)	0	++	++++	++++	++++	++++	++++	++(+)	++(+)	+	±
	1:20	+	±	++	++++	++++	++++	++++	+++	++(+)	++	+	0
	1:2	0	0	±	+	+(+)	±	±	0	0	0	0	0

Rabbit 5, weight 2,200 gm.

Precipitinogen.	1:20,000		0	0	0	0	0	0					
	1:2,000		0	0	0	0	0	0					
	1:200		+	+	0	0	0	0					
	1:20		++	±	+	0	0	0					
	1:2		++(+)	++	++	+	0	0					
Precipitin.	1:200,000		0	0	0	0	0	+					
	1:20,000		0	0	+	+(+)	+	+					
	1:2,000		0	0	+	++(+)	+	+					
	1:200		0	0	0	++	+(+)	+(+)					
	1:20		0	0	0	±	0	0					
	1:2		0	0	0	0	0	0					

Rabbit 16 was prepared by three intravenous injections of 0.5, 1, and 1.5 cc. of horse serum at intervals of 4 days. The last of the immunizing injections was given 25 days before the reinjection with 6 cc. of horse serum. Rabbit 5 was a normal control having had no prior immunizing injections. The normal control disposed of circulating antigen more rapidly than the immunized animal with circulating antibody.

TABLE V.

Antigen or antibody.	Final dilutions.	Before reinjection.	Time after reinjection (6 cc. of horse serum intravenously).							
			1 hr.	1 day.	2 days.	3 days.	5 days.	7 days.	8 days.	9 days.
Rabbit 16, weight 2,850 gm.										
Precipitinogen.	1:20,000	0	±	0	0	0	0	0	0	0
	1:2,000	0	+	+	+	±	0	0	0	0
	1:200	0	++	++	+(+)	+	+	0	0	0
	1:20	0	++(+)	++(+)	++	++(+)	+	0	0	0
	1:2	0	+++	+++	++(+)	+++	+(+)	±	0	0
Precipitin.	1:2,000,000	0	0	0	0	0	0	0	0	0
	1:200,000	0	0	0	0	0	+	+(+)	±	+(+)
	1:20,000	0	0	0	0	0	+(+)	++(+)	+(+)	++
	1:2,000	0	0	0	0	0	++(+)	+++	++(+)	+++
	1:200	0	0	0	0	+	+++	++++	+++(+)	++++
	1:20	0	0	0	0	+	++++	++++	++++	++++
	1:2	0	0	0	0	0	++++	++++	++++	++++

A description of the experimental procedure with this rabbit is given in the text (p. 498).

3. Sudden and Marked Diminution of Precipitin Following the Intravenous Injection of Antigen.—In all the rabbits which had circulating precipitin at the time of the reinjection there was a sudden and marked drop in the demonstrable precipitin in the serum during the 24 hours following the reinjection. The rapid diminution was followed by a prompt reaccumulation, so that 72 hours after the antigen injection the precipitin had returned almost or quite to the titer before injection. This seems to be too rapid a reaccumulation to be accounted for on the basis of newly formed antibody, even considering the fact that these animals have previously been immunized, and would therefore form antibody more rapidly than unimmunized animals.

4. Progressive Increase in Prozone as Antigen or Antibody in the Circulation Approaches the Time of Disappearance.—This observation was also made in following the time and manner of disappearance of antigen and antibody in serum disease. Observations were not made on all the rabbits for a sufficiently long time to determine just how frequently the prozone becomes progressively wider as antigen or antibody approaches the time when it ceases to be demonstrable in any dilution, but this tendency was noteworthy in Rabbits 3, 8, 10, 13, 14, 15, and 17. The tendency is more obvious in the disappearance of precipitin than precipitinogen. A good example of this is shown in Table VI. From this observation it is clear that the highest dilution at which a positive reaction is obtained does not give an accurate indication of the amount of antibody present.

In order to test further and in a different way the question of the relation of circulating antibody to the rate of disappearance of antigen the conditions of the experiment were altered. Two normal rabbits were injected intravenously with 6 cc. of horse serum. 15 minutes later they were bled for determination of precipitinogen. Immediately after the bleeding 12 cc. of undiluted anti-horse rabbit serum having a titer of 1:200,000 were injected intravenously. 30 minutes later they were bled again for determinations of precipitin and precipitinogen. Bleedings and titrations were repeated 24, 48, 72, and 96 hours later. It was found that 30 minutes after the injection of the antiserum none was demonstrable in the serum, and there was, moreover, no noticeable diminution in the amount of pre-

cipitinogen demonstrable in the circulation. 72 hours after the injection of the antiserum there was just as much precipitinogen in the circulation as before the antiserum was injected. The experiment was then repeated but much larger quantities of anti-horse rabbit serum were injected. In this experiment approximately 100 cc. of undiluted anti-horse rabbit serum were administered in the 48 hours following the injection of 6 cc. of horse serum. The results of this experiment are shown in Table VII. Unfortunately, one of the rabbits died on the 2nd day. It is noteworthy that the first 20 cc. of antiserum injected disappeared entirely from the

TABLE VI.

Final dilutions.	Precipitin in circulation.											
	May 14	15	16	17	18	19	21	23	25	28	31	June 4
Rabbit 22, weight 2,250 gm.												
1:200,000	0	0	0	0	+	0	0	+	0	0	0	0
1:20,000	+	±	0	0	+	+	+	+	+	+	+	0
1:2,000	+	+	+	+	+	+	+	+	+	+	±	0
1:200	+(+)	+	+	+	+	+	+	+	±	0	0	0
1:20	++	+	+	+	+	±	±	0	0	0	0	0
1:10	++	+(+)	+	+	+	±	0	0	0	0	0	0
1:2	±	0	0	0	0	0	0	0	0	0	0	0

On May 12, 1921, Rabbit 22 was injected intravenously with 6 cc. of horse serum and then, during the following 48 hours, was given intravenously 97 cc. of anti-horse rabbit serum. The above table illustrates the disappearance of precipitin from the circulation by progressive increase of the prozone.

circulation in 20 minutes, with practically no alteration in the amount of circulating precipitinogen; 15 minutes after the third injection of antiserum, with a total of 59 cc. injected up to this time, there was still no free precipitin, and the precipitinogen had diminished with no greater rapidity than may often be observed in normal rabbits injected with the same amount of horse serum and no antiserum. 17 hours after the fourth injection of antiserum (18 cc.) there were only slight traces of precipitin in the circulation. Despite the large amount of precipitin injected the precipitinogen was demonstrable till the 7th day after it was administered.

TABLE VII.

Antigen or antibody.	Final dilutions.	May 12					13				14			15	16	17	18	19	21	23
		9.40 a.m.	9.55 a.m. Bled.	10.00 a.m.	10.20 a.m. Bled.	4.45 p.m.	10.00 a.m. Bled.	10.15 a.m.	10.35 a.m. Bled.	4.45 p.m.	10.00 a.m. Bled.	10.15 a.m.	10.35 a.m. Bled.							
Rabbit 22, weight 2,250 gm.																				
Precipitinogen.	1:200,000	6 cc. of horse serum intravenously.	+	20 cc. of anti-horse rabbit serum (No. 23) intravenously.	±	19 cc. of anti-horse rabbit serum (No. 24) intravenously.	0	20 cc. of anti-horse rabbit serum (No. 21) intravenously.	0	18 cc. of anti-horse rabbit serum (No. 25) intravenously.	0	20 cc. of anti-horse rabbit serum (No. 26) intravenously.	0	0	0	0	0	0	0	0
	1:20,000		+		+		0		0		0		0	0	0	0	0	0	0	0
	1:2,000		++		+		+		+		0		0	0	0	0	0	0	0	0
	1:200		+++		++(+)		++		+(+)		+		±	0	0	0	0	0	0	0
	1:20		+++		++++		+++		+++		++(+)		++	+(+)	+(+)	+	+	0	0	0
	1:2		+++		++++		++++		++++		+++		+++	+++	++(+)	++	+	0	0	0
Precipitin.	1:2,000,000		0		0		0		0		0		0	0	0	0	0	0	0	0
	1:200,000		0		0		0		0		0		0	0	0	0	+	0	0	+
	1:20,000		0		0		0		0		0		+	±	0	0	+	+	+	+
	1:2,000		0		0		0		0		+		+	+	+	+	+	+	+	+
	1:200		0		0		0		0		±		+(+)	+	+	+	+	+	+	+
	1:20		0		0		0		0		0		++	+	+	+	+	±	+	0
	1:2		0		0		0		0		0		±	0	0	0	0	0	0	0

Rabbit 22 was a normal rabbit; no previous immunization. On May 12, 1921, it was injected intravenously with 6 cc. of horse serum. Following this, as shown in the above table, precipitin and precipitinogen were titrated at frequent intervals; anti-horse rabbit serum was introduced into the circulation in the amounts and at the times shown in the table. The precipitin titers of the five antiserums used were as follows: Rabbit 23, 1:200,000; Rabbit 24, 1:20,000; Rabbit 21, 1:20,000; Rabbit 25, 1:200,000; and Rabbit 26, 1:20,000.

TABLE VIII.

Antigen or antibody.	Final dilutions.	Dec. 7					8				9				10	11	12	13	14	15	16	17	18	19
		10.30 a.m.	10.45 a.m. Bled.	10.50 a.m.	11.10 a.m. Bled.	4.30 p.m.	9.30 a.m. Bled.	9.35 a.m.	9.50 a.m. Bled.	4.30 p.m.	12.30 p.m. Bled.	12.45 p.m.	5.30 p.m. Bled.	5.40 p.m.										
Rabbit 27, weight 2,100 gm.																								
Precipitinogen.	1:200,000	6 cc. of horse serum intravenously.	0	22 cc. of anti-horse rabbit serum (No. 28) intravenously.	0	14 cc. of anti-horse rabbit serum (No. 29) intravenously.	0	35 cc. of anti-horse rabbit serum (No. 7) intravenously.	0	15 cc. of anti-horse rabbit serum (No. 30) intravenously.	0	18 cc. of anti-horse rabbit serum (No. 31) intravenously.	0	22 cc. of anti-horse rabbit serum (No. 32) intravenously.	0	0	0	0	0	0	0	0	0	0
	1:20,000		±		0		0		0		0		0		0	0	0	0	0	0	0	0	0	0
	1:2,000		+		+		+ +		+		0		0		0	0	0	0	0	0	0	0	0	0
	1:200		++		++		+ +		+		+		± (+)		0	0	0	0	0	0	0	0	0	0
	1:20		++(+)		++(+)		++		++		++		+(+)		+(+)	+	+	+	±	±	0	0	0	0
	1:2		++(+)		++(+)		++(+)		+++		++(+)		++(+)		++	++	++	++	+(+)	+	±	±	0	0
Precipitin.	1:2,000,000		0		0		0		0		0		0		0	0	0	0	0	0	0	0	0	0
	1:200,000		0		0		0		+ +		0		0		0	0	0	±	0	0	0	0	0	0
	1:20,000		0		0		0		+ +		0		0		0	±	±	+	±	±	0	±	±	±
	1:2,000		0		0		+ +		+ +		±		±		+	+	+	+	+	+	+	+	+	+
	1:200		0		+		+ +		+(+)		+ +		+(+)		+(+)	+	+(+)	++	+(+)	+(+)	+(+)	+	+	+
	1:20		0		+		+		+ +		+ +		++		+(+)	+(+)	+(+)	++	+	+	+(+)	+	+	0
	1:2		0		±		0		±		±		±		±	±	±	±	0	0	0	0	0	0

Rabbit 27 was a normal rabbit; no previous immunization. On Dec. 7, 1921, it was injected intravenously with 6 cc. of horse serum. Following this, as shown in the above table, precipitin and precipitinogen were titrated at frequent intervals; anti-horse rabbit serum was introduced into the circulation in the amounts and at the times shown in the table. The precipitin titers of the six antiserums used were as follows: Rabbit 28, 1:500,000; Rabbit 29, 1:20,000; Rabbit 7, 1:200,000; Rabbit 30, 1:20,000; Rabbit 31, 1:500,000; and Rabbit 32, 1:500,000.

A repetition of this experiment with a still larger amount of antiserum injected into the circulation is shown in Table VIII. There is here an essentially similar result. In this instance the rabbit received 126 cc. of potent antiserum in the first 55 hours after the intravenous injection of 6 cc. of horse serum. Despite such a flooding of the circulation with the precipitin specific for the circulating antigen the latter was in the circulation in demonstrable amounts for 9 days and did not show a more rapid diminution during the period when the antiserum was being administered than is often seen during this period in normal rabbits injected only with horse serum. In this rabbit the first antiserum injected did not disappear entirely from the circulation as was the case with Rabbit 22 (Table VII).

By reference to Table I it is evident that the rate of disappearance of antigen in Rabbits 22 and 27, despite the addition of large amounts of precipitin to the circulation, is within the limits of variation of normal rabbits injected with the same amount of horse serum and receiving no antiserum. With such evidence it is justifiable to conclude that the intravascular union of precipitin and precipitinogen must play an insignificant part in removing precipitinogen from the circulation.

SUMMARY AND CONCLUSIONS.

1. In rabbits previously immunized to horse serum the rate of disappearance of horse serum after reinjection is somewhat more rapid than in control animals not previously immunized. But when the average duration of the persistence of antigen in ten previously immunized rabbits is compared with the average duration of persistence in ten normal controls, the difference is not impressive. This holds true for animals previously immunized by repeated injections but having no free circulating antibody, as well as for animals having a high titer of antibody at the time of reinjection. The same animal may dispose of circulating antigen as rapidly at a time when the blood contains no precipitin as when there is a high titer of circulating precipitin.

2. The intravenous injection of large amounts of potent anti-horse rabbit serum immediately after an injection of horse serum does not materially accelerate the rate of disappearance of the horse

serum from the circulation. While the number of days that circulating antigen was demonstrable in two experiments of this type was less than the average of controls the duration of persistence was within the limits of variation of normal controls.

3. Under the condition of the experiments intravascular union of antigen and antibody is an unimportant factor in the mechanism for disposal of antigen. By exclusion it therefore seems probable that the rate of disappearance of antigen is largely dependent upon cell avidity for the antigen.

4. Rabbits show a wide range of individual variation in the rapidity with which foreign serum is appropriated by the tissue cells, and in their ability to form precipitins. There does not appear to be any close relation between the amount of precipitin set free in the circulation and the rate of disappearance of precipitinogen.

5. The type of interrelation between precipitin and precipitinogen —demonstrable not infrequently in patients with serum disease—in which the precipitinogen diminishes rapidly when the precipitin rises to the crest of its curve is sometimes reproduced in rabbits. The type of curve which has been observed in patients with little or no serum disease in which the precipitinogen persists steadily in the circulation for many weeks and little or no antibody is formed, has not been observed in the present series of rabbits.

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EXPERIMENTAL STUDIES ON INFLAMMATION.

II. Experimental Chemical Inflammation in Vivo.

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(Received for publication, September 5, 1922.)

In a previous paper the influence of various chemicals upon chemotaxis, *i.e.* migration of cells *in vitro*, was discussed.[1] In the present paper the capacity of these chemicals to produce the other features of inflammation, *i.e.* vascular changes, migration of cells *in vivo*, fluid exudation, and tissue repair, will be considered.

A review of the literature on the subject of experimental inflammation reveals little that throws light on the main problem; namely, what physical, chemical, and physicochemical properties determine that a given substance will or will not produce an inflammatory reaction. No systematic classification has been made of the different substances in regard to their ability to arouse inflammation. Most of the articles have been descriptions of the different cell forms and types of exudates occurring in inflammatory reactions. No definite distinction has usually been made between aseptic and microbic inflammation.

Sollmann[2] alone has attempted to compare chemical structure with a related process, urticaria, but was unable to establish any relation between chemical constitution and urticarigenic action.

Ikeda[3] tested many different substances injected by Cohnheim's method into the frog. He found that normally a certain number of cells migrates into the exposed mesentery from the intact blood vessels within ¾ to 1½ hours, and those substances which decreased the number of cells were therefore considered as inhibiting to inflammation, those increasing the number as arousing inflammation.

[1] Wolf, E. P., *J. Exp. Med.*, 1921, xxxiv, 375.

[2] Sollmann, T., *J. Pharmacol. and Exp. Therap.*, 1916–17, ix, 391.

[3] Ikeda, Y., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 101, 137.

The following substances were found to inhibit the cell migration produced by exposure to air: quinine, atophan, mercury perchloride, arsenic, antipyrine, morphine, chloral hydrate, and calcium chloride. There was a slight inhibition when urethane and ethylhydrocupreine were injected. There was no inhibition produced by cinchonine, scopolamine, atropine, strophanthin, colchicum, sodium salicylate, and magnesium sulfate.

The rest of the abundant literature on experimental inflammation, including the extensive studies of Cohnheim, Marchand, Maximow, and others, reveals nothing of significance in relation to our particular problems.

In the present work experimental inflammation in both frogs and mice has been studied.

Method.

Method Employed with Frogs.—Medium sized winter frogs were used, being kept in a cage so designed that there was a constant stream of fresh cold water through part of the cage, and Cohnheim's method with the frog mesentery was employed. The different substances examined were injected into the dorsal lymph sac before pithing the frog, or were dropped on the exposed mesentery after pithing. Both methods were tried with each substance to serve as a possible check.

As a preliminary test of the behavior of substances introduced into the dorsal lymph sac, colored oils, scarlet R in olive oil and Sudan III, were injected into this cavity. The colored drops could be seen on the exposed mesentery 5 to 10 seconds after injection. Methylene blue solution is visible 3 to 5 seconds after injection. Therefore dorsal lymph sac injection is practically the same as intraperitoneal injection, the fluid passing directly from the sac to the peritoneal cavity by way of lymphatic spaces. In the routine work the first examination was made 30 minutes after injection. If there was evidence of inflammation at this time, examinations were made on other frogs at the end of 20, 10, 5, and even 2 minutes after dorsal injection. If the reaction was very slight or absent at the end of 30 minutes, another frog was examined 1 hour after injection. If there was no evidence of inflammation at this time, the substance injected was arbitrarily classified as one producing no inflammation. Normally from three to five cells per low power field will migrate from the blood vessels into the exposed mesentery in ¾ to 1 hour. In warm weather there may be ten or twelve, and when the surrounding atmosphere is very dry and no attempt is made to moisten the mesentery, this may increase to twenty cells per field. The strength of the solutions varied from 0.005 to 20 per cent molecular concentration of the various substances when the formula was known, otherwise the concentrations were percentage by weight.

Method Employed with Mice.—White mice of medium size, weighing about 25 gm., were injected intraperitoneally under aseptic precautions. Each mouse was kept in a separate cage after injection and the different animals were ex-

amined at the end of 1, 12, 24, and 48 hours respectively, depending upon the reaction to the injected substance. At the end of these periods the animal was anesthetized with ether, and kept under surgical anesthesia throughout the examination. After surgical preparation a midline incision was made into the peritoneal cavity; this was opened carefully to avoid injuring the intestines. The intestines were minutely examined for gross lesions, the mesentery for abrasions, areas of dullness, or changes in color or other abnormalities. The different regions were then examined microscopically in the same way as the frog mesentery. It was impossible to make quantitative determinations of the increase in the number of cells in the mesentery and around the blood vessels because of the numerous cells present normally. Qualitative and comparative differences could be easily determined. During examination the mice could be kept under ether anesthesia for several hours if necessary, and the condition of the heart and the respiration carefully watched. The mesentery was kept moist with sterile physiological salt solution warmed to 37°C., when necessary. In all cases 0.25 cc. of a 1 per cent solution (molecular concentration when the formula was known, otherwise concentration by weight) was injected. When other concentrations were used, they are noted.

EXPERIMENTAL.

Sodium Salts.—The Na ion produced no inflammatory reaction *per se*, being tested as the chloride, tartrate, lactate, phosphate (mono-, di-, and tribasic), citrate, bicarbonate, and sulfate. Sodium cinnamate produced a very slight inflammatory reaction in both frogs and mice, if used in strong (15 per cent molecular) solution. Tribasic sodium phosphate caused some inflammation by virtue of its alkalinity. In frogs, sodium iodide caused a fluid transudate when concentrations over 5 per cent were used, but there were only a few cells outside the blood vessels.

Potassium Salts.—These had little more effect than the corresponding sodium salts, and no inflammatory action can be attributed to the K ion. The iodide caused more hyperemia than the other potassium salts, and in mice a slight leucocytic migration was observed, but not in frogs. The other salts, when concentrated (15 per cent molecular), caused some fluid effusion and a slight hyperemia.

Calcium Salts.—No calcium salt caused a marked inflammatory reaction. In frogs, calcium chloride caused a slightly increased blood flow in 25 minutes in concentrations of 10 to 25 per cent, and calcium citrate produced a slight migration of cells after 20 minutes, if concentrations of 10 per cent or over were used. When a 3 per

cent solution was injected into mice there was marked slowing of the blood stream, and in concentrations of 6 to 20 per cent the mice died of convulsions in 10 to 20 minutes. There were no gross lesions of inflammation at this time, and microscopic examination showed a normal mesentery.

Calcium carbonate, tartrate, and cinnamate caused only foreign body reactions. There was some hyperemia in mice injected with calcium lactate, while citrate caused some slowing of the blood and diapedesis, but no leucocytic migration. Calcium cyanide caused no inflammation when 0.25 cc. of a 0.3 per cent solution was injected; when larger amounts or higher concentrations were injected, the mice died within 10 to 15 minutes with no evidences of inflammation.

Magnesium Salts.—None of the magnesium salts caused any change in the frog mesentery, except the citrate; this caused an active hyperemia. In mice a slight hyperemia was observed with strong solutions.

Strontium Salts.—Strontium chloride caused no inflammation in the frog mesentery, but in mice there were some hyperemia and a slight cellular increase. When equal parts of strontium chloride and magnesium sulfate were used together, there was a slight inflammation as shown by hyperemia and three to seven cells per low power field in $\frac{1}{2}$ hour in frogs. Similar slight effects were seen in mice, exceeding always the effects of either salt alone.

Barium Salts.—Barium sulfate and chloride caused no inflammation. When the solid particles were allowed to remain on the mesentery for several hours there was the typical reaction to a foreign body; *i.e.*, accumulation of cells around the foreign substance.

Mercury Salts.—Mercuric chloride caused no inflammation when a 0.5 or 1 per cent solution was injected into mice. When a 3 per cent solution was used there was a decided shrinking of the cells around the blood vessels and the mesentery was streaked with fibrin, but there was no true inflammation at the end of 24 hours. In higher concentrations the animals died within 24 to 36 hours with no evidences of mesenteric inflammation. Mercurous nitrate caused a very slight inflammatory reaction at the end of 1 hour; after 24 hours the mesentery looked red grossly and on microscopic examination there were many leucocytes, round cells, fibroblasts, and large oval or round cells with many nuclei. Mercuric subsalicylate produced no inflammation.

Free Organic Acids.—Lactic acid, nucleic acid, and uric acid caused no inflammation.

Oleic acid caused a decided dilatation of the intestinal and mesenteric vessels when a 5 per cent solution was injected in mice. The mesentery appeared cloudy, grossly. Microscopically there were fibrinous strands deposited around the blood vessels and an increase in the number of cells in the mesentery.

Amino-Acids.—In mice, glycine produced a slight inflammation at the end of 1 hour. With alanine the rate of flow was decreased but there were a few more cells free in the mesentery than normal. Phenylalanine, glutaminic and aminovalerianic acids, and tryptophane caused a slight increase in the number of polymorphonuclear leucocytes in the mesentery. Histidine caused no inflammation, but there was some diapedesis of erythrocytes. Tyrosine hydrochloride caused a dulling of the mesentery on injection; microscopically there were masses of white blood cells infiltrating fibrinous strands, but there was no increase in the amount of blood present. None of these amino-acids caused any inflammatory reaction in frogs.

Tyramine.—This compound was used in concentration of 1 per cent.[4] It was neutralized with 0.01 N NaOH, with phenolphthalein as an indicator (pH 7.7). In frogs it caused no true inflammation, but the white blood cells were clumped together in all the mesenteric vessels in small groups, at the end of about ½ hour, and there were red blood cells caught with the white blood cells, forming thrombi. The rate of flow of the blood was about normal for 25 minutes after injection, then the rate decreased, and became sluggish, and finally stopped. This clumping of the erythrocytes had previously been observed *in vitro*[1] and at that time no explanation could be given. It seems to affect the blood of different species in the same manner, in the *in vitro* work at least.

In mice, tyramine produced marked changes in the mesentery when 0.5 cc. of a 0.95 per cent solution of the hydrochloride was injected. Grossly the blood vessels appeared dilated at the end of 45 minutes. Microscopically there were groups of red blood cells clumped together within the blood vessels, particularly at the bifurcation of the smaller

[4] The tyramine was made synthetically and was provided by Dr. M. T. Hanke and Dr. K. K. Koessler.

veins, though there were some within the arterioles. In the small capillaries there was no circulation of the blood because of thrombi, although occasionally a thrombus would break loose and some few cells pass through, soon to be stopped by another clump of red blood cells. There were a few more extravascular cells than usual, but only the amount found in slight inflammation. At the end of 24 hours the capillaries showed only masses of clotted erythrocytes and there was no circulation in these vessels. In the mesentery there were some young connective tissue cells, some fibrin, and a few polymorphonuclear leucocytes. 48 hours after injection the flow of blood was still more sluggish than normal because of clots present at the bifurcation of the smaller vessels and in some of the capillaries. These were no longer the agglutination thrombi seen earlier but true clots with fibrin, red blood cells, and white blood cells. The mesentery was approximately normal, but there were more fibroblasts than normal and some early connective tissue cells. This is similar to the results obtained with frogs, although in these the predominating cell in the clumps of cells were white blood cells. With the mouse the clot was composed almost entirely of erythrocytes, white blood cells and fibrin being present only late, and in small amounts.

Histamine.—In concentrations of 0.1 to 0.25 per cent histamine caused marked inflammation when injected into the dorsal lymph sac of frogs. A set of observations is given.

9.00 a.m. Injection of 1 cc. of 0.25 per cent solution of histamine.

9.02 a.m. Increased rate of flow with an occasional white blood cell outside the contracted blood vessels.

9.05 a.m. Blood vessels are less contracted, from nine to twenty white blood cells outside the blood vessels per low power field.

9.10 a.m. More cells are outside the blood vessels, from twenty to twenty-five per low power field, the blood vessels are distended, and the rate of flow is increased.

9.15 a.m. White blood cells are scattered through the entire mesentery, some erythrocytes can be seen leaving the small capillaries by diapedesis, and the rate of flow is increased.

9.20 a.m. Frog died; heart stopped beating.

When histamine was applied directly to the frog mesentery the results were similar to those obtained on injection. Stained specimens made at different times showed deposition of fibrin strands

throughout the mesentery, and a thick layer of fibrin covered the mesentery at the end of 10 minutes, with many leucocytes and erythrocytes in the meshes. This action was in keeping with the marked positive chemotaxis that histamine showed in all the *in vitro*[1] work, when low concentrations were used.

In mice, histamine produced an inflammatory reaction at the end of 1 hour when 0.25 cc. of a 0.25 per cent solution was injected. It was found at the end of 15 minutes that the blood vessels were smaller than normal, and on microscopic examination the lumen was much reduced in size, much more so than the gross appearance of the vessel suggested. There were no evidences of inflammation at this time. 45 minutes after injection the mesentery appeared red, and the blood vessels were distended. Microscopically there were some few cells free in the mesentery, many of them being erythrocytes. These were most numerous around the smaller blood vessels, and some were in clumps around the capillaries. At the end of 1 hour, there were many more wandering cells free in the mesentery than before, and there was marked diapedesis of red blood cells. At the end of 24 hours, there was no acute inflammation but there were fibroblasts present throughout the mesentery, and a few polymorphonuclear leucocytes and some erythrocytes. At the end of 48 hours, there were still a few groups of polymorphonuclear leucocytes and an increase in the fibroblasts and young connective tissue cells. There were areas where erythrocytes had accumulated outside the blood vessels; the cell outlines were indistinct and broken. This is to be expected from the results obtained in frogs, although with frogs the reaction was more rapid and severe. *In vitro* work with histamine showed that the leucocytes of different animals react differently with histamine even under the same conditions, so that the present results are not surprising.

Since this work was done, the report of Bloom[5] has been published. He concludes that histamine does not produce inflammation when injected in cats, though there is a transudate which is most marked at the end of 1 hour. In mice the maximum action occurred at the end of an hour. When collodion capsules containing histamine, 1:100 in physiological saline, were placed between the internal and external oblique muscles in cats, there was a marked inflammatory

[5] Bloom, W., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 185.

reaction, but this was in no way different from that caused by collodion capsules containing normal saline, though very different from that caused by collodion capsules containing lactic acid, which is negatively chemotactic. When using open glass tubes containing histamine, *in vitro* and *in vivo*, Bloom found no attraction of leucocytes by the histamine; *i.e.*, no positive chemotaxis. His findings are contrary to those obtained by us;[1] and Sollmann[2] found histamine to be one of the most active agents (1:500,000) in producing urticaria, which is a process closely related to inflammation.

Alkaloids.—None of the following alkaloids caused inflammation: morphine and morphine sulfate, codeine and codeine hydrochloride, quinine sulfate and bisulfate, strychnine sulfate, brucine sulfate, caffeine sulfate and citrate, heroine and heroine sulfate.

All quinine salts caused a slowing of the blood stream and fewer cells migrated into the mesentery from the blood vessels 1 hour after exposure to the air, but the difference was so slight that it is impossible to say that quinine inhibits inflammation.

Ethyl Alcohol.—No inflammation was caused in mice in concentrations below 10 per cent, but in concentrations of 5 to 25 per cent there was a slight migration of white blood cells. In concentrations of 25 to 30 per cent there were many white blood cells outside the blood vessels, from seven to twenty per low power field. These were smaller than normal, and the entire mesentery appeared dry. When fixed and stained, there were strands of fibrin present and the mesentery appeared dehydrated.

Ethyl Acetate.—This compound acted like ethyl alcohol, the inflammation being negligible. It was used as a solvent for cantharidinum.

Ether.—Ether acted like ethyl alcohol, but a 1 per cent concentration acted like the 10 per cent solution of alcohol. There was marked dehydration of the frog omentum in higher concentrations.

Urotropine.—Urotropine, or hexamethylenamine, caused a mild inflammation in frogs with concentrations of 5 per cent at the end of $\frac{1}{2}$ hour if the media was acid. When the mesentery was neutral or alkaline there was no reaction.

Parazol.—This substance (crude dinitrodichlorobenzene)[6] in concentrations of 1 per cent or less caused a slowing of the blood stream of the frog omentum and then in many instances the heart stopped

[6] Wells, H. G., *J. Indust. Hyg.*, 1920, ii, 247.

beating. There were a few white blood cells outside the blood vessels. Stained specimens showed a marked deposition of fibrin through the mesentery, and the cells were indistinct, with poorly stained nuclei. In mice severe typical inflammation was produced by 0.2 cc. of a 0.3 per cent aqueous or oily solution, accompanied by much necrosis. This is somewhat similar to the action of parazol *in vitro*, in which high concentrations cause a negative reaction (chemotaxis) with a necrotizing action, while low concentrations are positively chemotactic.

Papain.—Papain, or papayotin, acted differently in winter and in summer frogs. In the former there was no inflammation when a 20 per cent solution of papain was injected into the dorsal lymph sac or when it was applied directly to the exposed mesentery. When summer frogs were used or when winter frogs were kept in a warm place for several hours, so that the body temperature was over 85°F., there was a definite inflammation on the injection of a 20 per cent solution of papain. This is similar to the results obtained *in vitro*, when the blood of summer or winter frogs was used, the former being positively chemotactic, the latter negatively chemotactic.

In mice, papain, or papayotin, caused a slight inflammation at the end of an hour. There were many white blood cells throughout the mesentery. At the end of 24 hours, the intestines were red, and the mesenteric vessels were distended. Microscopically there were many more wandering cells around the blood vessels than normal and ameboid movements could be seen. In the mesentery there were groups of cells, largely polymorphonuclear leucocytes, though some fibroblasts were present, and in the meshes of fibrin a few erythrocytes. At the end of 48 hours, there were many connective tissue cells with interfibrillar substance, and a few large cells with faintly staining nuclei, epithelioid cells, around the red blood cells. This was similar to the results obtained with summer frogs and is to be expected from the *in vitro* work with the blood of warm-blooded animals.

Cantharidinum.—Cantharidinum (U. S. P.), obtained from Chinese cantharides, caused marked inflammation in the frog mesentery. This drug was dissolved in 5 to 10 per cent ethyl acetate in water. The following is a typical protocol.

8.00 a.m. Injected 1 cc. of a 2 per cent solution of cantharidin into the dorsal lymph sac.

8.02 a.m. Rapid rate of flow of blood.

8.05 a.m. From two to five cells outside the blood vessels per low power field.

8.10 a.m. Marked distention of blood vessels; from nine to fifteen white blood cells per low power field.

8.12 a.m. Increasing number of white blood cells emerging from blood vessels.

8.20 a.m. Many more cells outside the blood vessels, from 70 to 100 per low power field; diapedesis of erythrocytes.

8.30 a.m. Too many cells outside the blood vessels to count; blood vessels contracting slightly.

8.40 a.m. Heart stopped beating.

All the mesenteries were stained and it was found that at the end of 5 minutes after injection the walls of the blood vessels were thinned and strands of fibrin could be followed along the blood vessels. At the end of 15 minutes, the fibrin was present in sheets and many polymorphonuclear leucocytes, red blood cells, and a few lymphocytes were free in the mesentery.

In mice, cantharidinum produced marked inflammation when 0.25 cc. of a 0.5 per cent solution was injected. The mice were unable to stand. Gross examination revealed a yellow exudate at the end of $\frac{1}{2}$ hour. Microscopically there were many polymorphonuclear leucocytes in the mesentery, and fibrinous strands along the blood vessels with erythrocytes in the meshes. At the end of 1 hour, the entire mesentery was covered with polymorphonuclear leucocytes and erythrocytes, and in some places the fibrin was so thick that the cells were hard to see clearly. At the end of 24 hours, there was no exudate present, but microscopically there were groups of polymorphonuclear leucocytes around clumps of fibrin in certain areas. There were many fibroblasts. At the end of 48 hours, the fibroblasts had increased markedly and the polymorphonuclear leucocytes had decreased, the fibrin was diminished, and there were only a few erythrocytes present. 72 hours after injection, there was little evidence of inflammation; the increase in fibroblasts and immature connective tissue alone showed that there had been an inflammation.

Croton Oil.—This substance caused severe inflammation. Some consider that the active principle of croton oil is an acid,[7] others

[7] Bastedo, W. A., Materia medica; pharmacology; therapeutics; prescription writing, for students and practitioners, Philadelphia and London, 2nd edition, 1918, 136.

that it is a resinous anhydride. When 0.25 cc. of a 1 per cent solution in olive oil was injected into mice there was no inflammation at the end of 1 hour, but within 6 hours there were many white and red blood cells in the mesentery and a marked increase in the rate of flow of the blood. At the end of 12 hours, the entire mesentery and intestines were red, and in places the peritoneum was no longer smooth and shining, though there were no definite areas of exudation. There were many cells in the mesentery. At the end of 24 hours, the individual oil drops were surrounded by numerous cells and in many instances by fibrinous strands. There were a few fibroblasts at the outer edges of these clumps of cells. At the end of 48 hours, there were many fibroblasts, many of the polymorphonuclear leucocytes had disappeared, and the oil drops were fewer in number and smaller in size. Young connective tissue cells were present, though not numerous. At the end of 72 hours, there were many fibroblasts and connective tissue cells, but few other evidences of inflammation. These cells were abundant around the traces of oil that were still left. When higher concentrations were used, there was more active, early inflammation, with more polymorphonuclear leucocytes, and many erythrocytes were present in meshes of fibrin.

In frogs similar inflammatory reactions were produced. With solutions over 10 per cent the frogs had convulsions in spite of pithing and at the end of 20 minutes the blood vessels to the mesentery were so torn that it was impossible to examine the tissues under the microscope. Stained specimens at the end of 5 minutes showed large amounts of fibrin in the mesentery and some few polymorphonuclear leucocytes.

Turpentine.—Marked inflammation was induced with concentrations of 0.5 to 5 per cent in olive oil. When higher concentrations were used in frogs the blood vessels ruptured before the animal could be examined. Stained specimens showed strands of fibrin along the blood vessels at the end of 10 minutes, and almost a complete sheet of fibrin over the mesentery at the end of 25 minutes. In mice typical inflammatory reactions were produced.

Jequirity.—A preparation made by grinding the jequirity seeds, adding water, and shaking in a shaking machine for 24 hours caused only a slight inflammatory reaction; indeed there was just about

the number of cells free in the mesentery that is usually found after exposure to air. This was repeated many times with seeds from three different sources, and the same results were obtained, despite the reputation this seed has for producing profound inflammatory changes.

Miscellaneous Substances.—Urethane, urea, creatinine, olive oil, veronal (diethylmalonylurea), yeast vitamine (Harris), olive oil, chloretone, glucose, mercurochrome 220, Witte's peptone, egg albumose, and Armour's peptone caused no inflammation in either frogs or mice. In mice, acetanilide caused a slight inflammation at the end of 8 hours.

The effect of aspirin (acetyl-salicylic acid) was peculiar when the usual amount, *i.e.* 0.25 cc. of a 1 per cent solution, was injected. The animals died within 10 minutes, with no lesions to account for death, and no inflammation was present. When 0.1 cc. was injected, there was no evidence of inflammation at the end of 24 hours.

Scarlet R, purified, caused no inflammation at the end of 1 hour, but 24 hours after injection, there were many erythrocytes and polymorphonuclear leucocytes around the individual oil drops. At the end of 48 hours, there were many fibroblasts, but no true inflammation.

DISCUSSION.

Cohnheim's method for the study of aseptic inflammation gives very satisfactory results for *in vivo* work. Definite quantities can be injected and a microscopic examination made of the mesenteries.

The results may be determined in a definitely quantitative manner by counting the cells migrating out of the blood vessels in a given field, and measuring the increase in size of the blood vessels by the fine screw on the microscope, when frogs are used. With mice, qualitative results only can be obtained; quantitative results are impossible, because of the normal cellularity of the mesentery.

The different types of cells can be detected easily without fixing or staining, but fixing with Zenker's fluid and staining with hematoxylin and eosin make the different cells more distinct; also the changes in the walls of the blood vessels and the amount of fibrin are striking. The migration of the leucocytes through the capillary walls can only be seen distinctly during the life of the animal, before fixation and the subsequent shrinkage.

Dorsal lymph sac injection in frogs is practically the same as intraperitoneal injection; this was clearly seen when colored fluids were injected, as a watery solution of methylene blue, or an oily solution of scarlet R, which make their appearance in the peritoneal cavity almost immediately.

The body temperature of the animal seems to be of importance with certain substances, particularly papain. In the majority of instances, however, it makes no difference. Substances which react differently at different temperatures *in vivo* are found to be those which reacted differently towards the blood of cold- and warm-blooded animals *in vitro*.

CONCLUSIONS.

1. None of the salts tested produce a marked inflammation *in vivo* in concentrations under 10 per cent. Potassium salts and the different citrates produced atypical inflammatory reactions in mice, but not in frogs. There was no true inflammation, however, characterized by blood vessel changes, migration of polymorphonuclear leucocytes and erythrocytes, and fluid exudation.

2. Synergistic action occurs when equal parts of strontium and magnesium salts are employed. There is a change in the appearance of the mesentery without a true inflammation, and this change does not occur with either salt alone.

3. Amino-acids and amines as a class do not produce inflammation, but histamine produces a marked inflammatory reaction in frogs and mice.

4. Tyramine does not cause an inflammatory reaction but has other marked effects; agglutination thrombi occur within the smaller blood vessels, both veins and arteries; in frogs there is a rapid clumping of the white blood cells followed by a true coagulation with strands of fibrin and entanglement of erythrocytes. This is very widespread and often kills the animal within an hour after injection. In mice it is the erythrocytes that clump and coagulation occurs very much later, usually at the end of 24 hours; still later there is complete absorption of the coagulated masses and the mesenteric circulation returns to normal. None of the mice died during the stage of clumping, and the clots never extended up the larger vessels as they did in the frogs.

These effects are similar to the phenomena observed in the *in vitro* work, in which clumping of the cells appeared constantly.

5. Cantharidinum, histamine, and turpentine produced the most rapid and marked inflammation of any substances tried. These substances are all strongly positively chemotactic *in vitro*. The differences occurring when these substances are used in different species is a quantitative rather than a qualitative one, the body temperature being of some importance. Papain acted only in warm-blooded animals; this is consistent with its chemotactic action *in vitro*. The degree of positive chemotaxis varied markedly with the blood employed and in the *in vivo* work the inflammation varied with the species of animal used.

6. Certain substances produced inflammation only some time after injection; this is true of scarlet R and croton oil in weak dilutions. These are not strongly positively chemotactic.

7. Parazol produces an inflammation associated with necrosis of the tissues. This is similar to the results obtained *in vitro*, parazol being positively chemotactic in low concentrations and negative in high concentrations.

8. The exact chemical nature of many of the substances which produce marked inflammation is unknown. This is true of cantharidin, and the active constituents of turpentine and croton oil.

9. All substances which produce marked and rapid inflammation on injection are positively chemotactic, but not all strongly positively chemotactic substances produce inflammation; *i.e.*, calcium compounds, sodium phosphate, etc.

10. Only substances which are positively chemotactic and also soluble in oil seem capable of producing inflammation in animals.

EXPERIMENTAL OBSERVATIONS ON THE PROPHYLAXIS AND TREATMENT OF SYPHILIS.

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PLATE 28.

(Received for publication, November 24, 1922.)

I.

INTRODUCTION.

Experimental syphilis in the rabbit is a subject which has developed considerably in the last decade. One of the most important advances has been the conclusive demonstration by Brown and Pearce that the disease in the rabbit is essentially a general one and that the spirochetes survive for an indefinite period in animals, once infected, as proved by the transfer of the disease with the tissue of lymphatic glands. This procedure forms an excellent check on clinical and microscopic examinations of the local lesions in the testicle and scrotum. On the other hand, the recently demonstrated existence of a natural rabbit syphilis, which at first seemed to threaten to invalidate the results of experimental work, has not proved a serious complication in the kind of experiments which we have used.

Our work here reported confirms the value of the present methods of prophylaxis.

The treatment of syphilis has made great advances since the introduction of arsphenamine, but there still seems to be a school which believes that infection with *Treponema pallidum* is incurable. The evidence for this position seems to be based on relapses following inadequate treatment, on positive gland transfers in latent cases, and on the finding of spirochetes, post mortem, in old cases which have also received indifferent treatment. The possibility of a granular stage in the life cycle of the organism has also been cited in favor

of this view. The following work has been undertaken in order, if possible, to throw some light on this subject, and as far as the experimental work goes, it shows that an infection can be entirely abolished by arsphenamine.

II.

Prophylaxis.

The first preparation to be used scientifically in the prophylaxis of syphilis was the now classical 33⅓ per cent calomel ointment of Metchnikoff. This was tested on monkeys with uniformly satisfactory results, and finally on the medical student, Maisonneuve, who offered himself for the experiment (1). The work of Metchnikoff, however, early encountered criticism, particularly from Neisser (2). Neisser's objections to calomel ointment are discussed at length by Vedder (3) in a review of the subject. Vedder calls attention to the fact that Neisser worked usually with 10 to 20 per cent calomel ointments, and that in the three instances in which he used 33⅓ per cent as recommended by Metchnikoff two of the animals were protected against syphilis. These results are at least as good as those he obtained with other antiseptics. Neisser believed that the incorporation of the antiseptic in a fatty base interfered with its action, and he, in collaboration with Siebert, recommends a paste composed of amylum, tragacanth gelatin, and mercuric chloride.

Schereschewsky (4) advocates a quinine ointment, and has tried this out practically with troops, with "satisfactory" results. His ointment is sold under the trade name of "Duanti." It apparently contains 40 per cent quinine (5), though we have not been able to locate a statement of its exact composition by Schereschewsky. He considers that calomel ointment has little spirocheticidal action and states that it does not cause the disappearance of spirochetes within 24 hours when applied to rabbit lesions containing numerous spirochetes (6). Neither did calomel ointment interfere with the motility of spirochetes when mixed with them on a slide. Quinine ointment, on the other hand, according to the same author, caused the disappearance of spirochetes from lesions, and also stopped their motility immediately in microscopic preparations. He (7) also states that quinine ointment applied 1 or 2 hours after intercourse prevents the transmission from animal to animal of the spontaneous spirochetosis occurring in rabbits. He did not test calomel ointment in this respect, since he considered that its uselessness had been fully demonstrated by other experiments.

Schumacher (8) has recently reviewed the question of prophylaxis, and takes up in detail several of the preparations that have been recommended for this purpose. He states that the calomel ointment of Metchnikoff possesses only historical interest, since all authors have agreed that it is without effect. He says emphatically that it should be removed for all time from the list of substances to be used in the prophylaxis of syphilis. To his mind the fact that calomel is practically insoluble precludes any possible effect, owing to the absence

of Hg ions. Some of the recent criticisms of calomel ointment are surprising when it is considered that Neisser, the first author to object to it, found it at least partially successful.

Calomel ointment has been used in the prophylaxis of syphilis in both the United States Army and Navy for a number of years. The results have been eminently satisfactory, according to Vedder's summary, and as stated in the reports of the Surgeon Generals of both Services. Its value seems to have been particularly demonstrated during the recent war, as has been brought out by Young (9, 10), G. Walker (11), and many others. The fact has been recognized that its efficiency diminishes rapidly as the time after exposure increases. The ointment used in the Army has the following formula.

Calomel	30 parts.
Benzoinated lard	65 parts.
White wax	5 parts.

The application of the ointment is preceded by preliminary cleansing with soap and water, and the injection of protargol (1 per cent) into the urethra. Regulations require that this prophylaxis be taken as soon as possible after exposure.

As a result of experience in France during the war, Dr. Hugh Young concluded that, if possible, the prophylaxis should be simplified by omitting the preliminary washing and by using a single antiseptic which would be effective against syphilis, chancroid, and gonorrhea. Calomel ointment alone is not effective against gonorrhea, although it can be made so by the addition of 3 per cent carbolic acid and camphor as in the old Army and Navy packet (12) and the present Navy tube. There is evidence that calomel treatment is also less effective against chancroids than against chancres (11). Dr. Young suggested the use of some of the more powerful mercurial antiseptics which have been developed at the Brady Institute such as "Meroxyl" or "253." The following work was undertaken as a basic study of present methods with a view to establishing a means of comparison with other antiseptics, which are now under investigation.

A. Method.

Most of the early work was done with monkeys, but as we wished to be able to use a great number of animals, we employed rabbits.

First some preliminary experiments were done in order to work out a method for their utilization. The application of a spirochete suspension to the unbroken skin does not result in an infection. The unbroken skin of the scrotum of two animals was inoculated with a heavy suspension of spirochetes; the animals were held under observation for 90 days, gland transfers being made at the end of 45 days. None of the animals became infected. However, if the skin over both testicles is scarified as in the old vaccination method, a high percentage of takes is obtained, 100 per cent when both testicles are considered and 81 per cent of individual testicles (Table I). This method was then combined with the gland transfer method, which yielded in the controls 87.5 per cent of takes. These two procedures afford a fairly accurate method of testing prophylaxis.

The strain of *Treponema pallidum* used was the so called Nichols (13) strain which was isolated from spinal fluid in 1912 and has been carried on in rabbits ever since. That it is particularly suitable for this sort of experimental work we are not prepared to state on the basis of rigid comparative tests, but it has been largely used in other experimental work and its characteristics are well known.

1. Local Inoculation.—The testicle was drawn into the scrotum and held in such a manner that the skin over it was taut. The hair was shaved without soap, and the surface sterilized with alcohol, which was allowed to evaporate thoroughly. From fifteen to twenty superficial abrasions were then made by allowing a razor to glide lightly over the surface of an area of approximately 1 by 1.5 cm., bleeding being avoided as much as possible. About 0.05 cc. of a spirochete suspension was placed on the scarified area and rubbed lightly with the sides of the capillary pipette. This was allowed to dry and the animal was replaced in its cage. Both testicles were invariably inoculated in the same way.

At fixed intervals the animals to receive prophylaxis were removed from the cages and approximately 1 gm. of ointment was rubbed in on each side with moderate pressure by means of a wooden spatula. The control animals, of course, received no prophylactic ointment.

The spirochete suspension was made by removing a small cylinder of tissue from a chancre of the scrotum with a large bore capillary pipette, or better, by removing a testicle containing syphilitic nodules[1] and cutting out the affected portions with scissors. The latter method gave much richer spirochete suspensions, and, as will be mentioned later, saved time on account of the more rapid development of lesions. The tissue removed was ground up in a sterile mortar, and from 1 to 2 cc. of salt solution were added. The addition of a small

[1] All operations were performed under ether anesthesia.

amount of sand or a few bits of broken glass aided in the grinding. It is inadvisable to add citrate solution, as this interferes with the motility of the organisms and distorts them somewhat. It does not interfere with their viability, however.

As Tables I and III show, the animals were inoculated in four groups at different times, and hence of necessity with different spirochete suspensions. The number of spirochetes in each suspension was estimated by counting microscopic fields. Since the suspensions varied in the number of spirochetes, an opportunity was afforded of determining whether the strength of the suspension affected the incubation period of the disease.

Groups 1 and 2 were inoculated from suspensions containing three to five spirochetes per field, Group 3 from a suspension containing one to three spirochetes per field, and Group 4 from a rich suspension obtained from testicular material containing six to thirteen spirochetes per field. Three controls were used with Group 1, one control with Group 2, and two controls each with Groups 3 and 4. The controls for Group 1 were positive in from 33 to 36 days, for Group 2 in 32 days, for Group 3 in 35 to 49 days, and for Group 4 in 16 to 17 days. Hence it seems that the rich suspension used for Group 4 shortened considerably the incubation time of the disease.

One of the controls for each group was invariably inoculated at the end of the experiment; that is, after all the other animals had been inoculated. This was done in order to be absolutely certain that the organisms were not injured by being kept at room temperature while the animals were being inoculated. All these controls were positive, showing that the organisms apparently were in no way injured. In Group 4 a period of 1 hour and 35 minutes elapsed between the inoculation of the first animals and that of the last control. Moreover, the organisms at the end of this time were still actively motile.

All animals, with the exceptions noted in the tables, were kept under observation for a period of at least 90 days. They were examined microscopically once a week, or oftener. Microscopic examinations with the dark field were made of animals that showed at the site of inoculation or of gland transfer any edema or thickening of the skin or subcutaneous tissue. The microscopic examinations were made by grasping the suspicious area between the blades of a pair of thumb forceps, and arranging the tissue in such a way that the blood was forced out, when the area appeared pale and distended by the subcutaneous fluid. The distended skin was then punctured and the drop of clear serum oozing out was collected by touching it with the surface of a cover-slip. This was immediately examined under the dark-field microscope. No animal was considered positive until proved so microscopically. The date of such proof is taken as the date of becoming positive. In practise it always coincided

with the first appearance of a characteristic initial lesion, which usually rapidly developed into a definite chancre.

The initial lesions consisted of a circumscribed area of from $\frac{1}{4}$ to $\frac{1}{3}$ cm. in diameter, showing slight edema, redness, and some thickening of the skin. The blood vessels leading into them were usually dilated. When presenting this characteristic appearance they never failed to be positive microscopically. In about a week they usually developed into pale button-like areas of induration, followed by ulceration and progressive enlargement of the chancre until it became knob-like and of a diameter of 1.5 to 2 cm. or more. As noted, however, some of the lesions did not progress this far.

A few of the animals developed a diffuse non-specific inflammation of the skin of the scrotum. The thickening and edema embraced not only the inoculated area, but practically the whole scrotum. The edema was sometimes marked. Despite the fact that the appearance was not at all characteristic, all animals showing it were repeatedly examined microscopically, with negative results. This inflammation was attributed to trauma from the shavings in the bottom of the cage, especially since the hair of the scrotum had been shaved away.

The size of the inguinal lymph glands was noted on all examinations. Their reaction was inconstant, as in some animals they were markedly enlarged, and in others with similar local lesions they were not palpable.

2. Gland Transfers.—In transferring glands from one animal to another, we varied somewhat the technique of Pearce and Brown (14). They removed the gland, ground it up in a mortar, and injected it into the testicular substance with a syringe. The variation we made consisted of removing the gland and placing it *in toto* in the subcutaneous tissue of the scrotum through a small opening made in the skin. The opening was then closed with collodium. This method left the gland in a superficial position, where any changes in it or in the surrounding tissue could be readily followed and easily examined microscopically. One of the inguinal glands was always used, being removed under ether anesthesia. Few suppurations occurred, and even when they did there was apparently no interference with the subsequent development of chancres. The gland transfers were made only after the controls had become positive, 28 to 72 days from the date of inoculation.

The animals receiving glands from cases clinically negative were without exception kept under observation for at least 90 days. As shown in Table III, they were all negative, and require no further comment.

B. Results.

Table I shows the results obtained with eight animals inoculated as controls, which received no prophylactic ointment. They were

TABLE I.

Control Animals. Rabbits Inoculated with Treponema pallidum by Scarification of the Skin of the Scrotum; No Prophylactic Ointment Applied.

Experiment No.	Rabbit No.	Group.	Result.		Day testicle became positive for spirochetes.	Remarks and description of lesions.
			Right side.	Left side.		
1	1	1	+	+	36	Bilateral indurated and ulcerated chancres 1.5 cm. in diameter. Lesions were regressing when animal was discarded on 90th day.
2	2	1	+	+	36	"Blank" ointment applied 1 hr. after inoculation. Bilateral indurated and ulcerated chancres. Complete disappearance of lesions by the 88th day.
3	3	1	−	+	33	Left side only positive; small initial lesion. Small suspicious lesion on right side, not positive microscopically. Animal died on 33rd day from pneumonia.
4	4	2	−	+	32	Left side only positive; small indurated chancre 1 cm. in diameter, disappearing completely by 67th day. Gland transfer made on 68th day positive.
5	5	3	+	−	35	Right side only positive; indurated ulcerated chancre 1 cm. in diameter. No lesion on left side (Fig. 1). Animal died of pneumonia on 58th day.
6	6	3	+	+	49	Small bilateral areas of localized edema and slight induration, disappearing in a few days. Gland transfer made on 41st day positive.
7	7	4	+	+	17	Large bilateral indurated and ulcerated chancres, 2.5 cm. in diameter. Chancres were regressing when animal was discarded on 90th day.
8	8	4	+	+	16	Large bilateral indurated and ulcerated chancres, 1.5 cm. in diameter. Animal sacrificed on 55th day for other experiments.

Of eight controls, 100 per cent were positive on one side or the other. 81 per cent of the individual testicles were positive.

100 per cent positive for syphilis in one testicle or the other and 81 per cent of the individual testicles were positive. One animal (No. 2) received an application of a "blank" ointment, consisting of a mixture

of equal parts of lanolin and vaseline. The ointment was applied 1 hour after inoculation. This was done to determine whether the base of the ointment alone has any effect, particularly since some authors have raised the question of whether the fatty base in itself might not have an inhibiting effect. In this experiment the base alone had apparently no effect, since the animal developed syphilis.

TABLE II.

Control Gland Transfers. Gland Transfers from Known Syphilitic Animals.

Experiment No.	No. of original rabbit.	Day of gland transfer.	No. of rabbit receiving gland.	Result of gland transfer.	Day gland transfer became positive for spirochetes.	Remarks and description of lesions in animals receiving glands.
1	6 (Table I).	41	15	+	41	Large chancre, 2 cm. in diameter, indurated and ulcerated. Lesions still present when animal was discarded on 90th day.
2	9	41	16	+	20	Large chancre.
3	4 (Table I).	68	17	+	77	Small area of edema and induration at site of gland transfer, disappearing in about 7 days.
4	10	105	18 (left side).	+	32	Large chancre.
5	11	170	18 (right ").	+	36	" "
6	12	231	20	+	47	" "
7	13	261	21	+	56	" "
8	14 (Table III).	30	22	−	−	No lesion after 90 days.

With gland transfers 30 to 261 days after inoculation, of eight animals, seven, or 87.5 per cent, were positive.

Rabbits 3 and 6 developed only small lesions. Both these animals, however, began to lose weight soon after beginning the experiment; one died on the 33rd day, and the other on the 49th day, after developing pneumonia that ran a rather chronic course. The pneumonia was due to an organism of the hemorrhagic septicemia group. Loss of weight in an animal showing lesions was usually accompanied by disappearance or regression of the lesions. The healthier the

animal, the more characteristic was the lesion. Hence, healthy stock is of great importance for the success of the inoculations as is a rich spirochete suspension.

TABLE III.

Animals Treated with 30 Per Cent Calomel Ointment at Varying Intervals after Inoculation.

Experiment No.	Rabbit No.	Group.	Interval before application of ointment.	Result.	Day of transfer.	Result of transfer.	Remarks.
			hrs.				
1	23	4	1	—	29	—	Original animal died of pneumonia on 35th day.
2	24	1	1	—	53	—	
3	25	2	2	—	44	—	
4	26	3	2	—	39	—	Original animal died of pneumonia on 39th day.
5	27	4	3	—	29	—	
6	28	3	3	—	41	—	
7	29	3	4	—	41	—	
8	30	3	4	—	78	—	
9	31	4	4	—	27	—	Original animal died of pneumonia on 27th day.
10	32	4	5	—	29	—	
11	33	4	5	—	37	—	
12	34	4	6	—	37	—	
13	35	4	6	—	28	—	
14	36	4	7	—	30	—	Original animal died of pneumonia on 63rd day.
15	37	4	7	—	37	—	
16	14	4	8	+ (36 days).	30	—	Left side only positive; small area of localized edema and slight thickening, rapidly disappearing. Right side showed slightly suspicious area, not positive microscopically.

All transfers were observed for 90 days.

Table II shows the results of eight control gland transfers from positive animals. Seven, or 87.5 per cent, were positive. The percentage is less than that of Brown and Pearce, but is sufficiently high to be of value when taken with the results of Table I.

Table III shows that in fifteen instances the calomel prophylaxis was successful when practised for periods up to 8 hours after inoculation. Neither the original animal nor the transfer showed the slightest evidence of infection. At 8 hours, the only animal treated developed a chancre. These results were clear-cut and convincing, especially when compared with the results in the controls, in the animal treated with blank ointment, and in the animals treated with other antiseptics.

C. Influence of Base Ointment.

Metchnikoff's original ointment was composed of ten parts of calomel and twenty parts of lanolin. Vedder (3), in reviewing the subject, likewise concluded that lanolin was the proper base for the calomel. As stated above, the Army has used for a number of years a base composed of lard and white wax.

We tested calomel in a base of lard and white wax, and also in a base of lanolin and vaseline. In Table III the animals are grouped according to the hour at which they were treated. Of each pair dealt with at a given hour one was treated with a lard and wax, and one with a lanolin and vaseline base, except at 4 hours, when one received the lard and wax base, and two lanolin and vaseline, and at 8 hours, when the single animal was treated with a lard and wax base. The result shows that, if there is any difference dependent on the base, it must be slight.

D. Death of Animals Due to Prophylaxis.

An example of the absorption of mercury and the sensitiveness of the rabbit to mercury occurred in five animals which died of mercurial nephritis following prophylaxis with calomel ointment. The animals were given an application on the scrotum of about 5 gm. of calomel ointment and died in 5 to 15 days. The only lesion found post mortem was in the kidney. The pathological report on one of these kidneys is as follows:[2]

"The kidneys show an advanced granular degeneration of all parenchymatous structures with fatty degeneration and necrosis of the type cells. Extensive deposits of lime salts in the convoluted tubules and Henle's loops typical of acute mercurial poisoning in man are found everywhere throughout the cortex."

[2] This report was kindly made by Major G. R. Callender, of the Army Medical Museum.

The animals dying were replaced by animals with which only 2 gm. of calomel ointment were used, and no further difficulty was experienced. In order to be absolutely certain that the deaths were due to calomel ointment, six normal animals were treated with from 5 to 15 gm. of calomel ointment. These animals all died with typical kidney changes in from 5 to 7 days. There was no difference between calomel in a lard base and calomel in a lanolin and vaseline base in this respect. These six animals were maintained for 48 hours in narrow inoculation boxes, which precluded the possibility of ingestion by licking.

It is surprising that an inert insoluble substance such as calomel should be absorbed so readily through the skin. The danger is thus shown of attempting to predict the physiological, and perhaps also the bactericidal, properties of a substance from its physical properties. Our observation in this particular confirms the previous work of Schamberg and his coworkers (15) who showed that inunctions of calomel ointment caused the death of rabbits. One of their animals died after two inunctions, each of 1.3 gm. of 50 per cent calomel ointment, given with a 5 day interval. Their animals, however, did not show the marked kidney lesions that were present in our animals that died following prophylaxis. As a result of their work they urged that inunctions of calomel ointment be used in the treatment of syphilis. Cole and Littman (16) tried this out clinically with results that were not satisfactory, though Schamberg, in discussing their paper, suggested that larger amounts of the ointment should be used.

III.

Treatment.

A. Method.

The problem was to test by the gland transfer method the sterilizing effect of arsphenamine and neoarsphenamine on rabbits infected with *Treponema pallidum*. For this purpose animals were selected which had had typical inoculation chancres and which had been infected long enough for a general infection to become thoroughly established. In other words, they were either latent or old cases in the human sense, with disease lasting from 3 to 6 months. They were treated with a different number of doses as indicated in Table IV and at

intervals from 28 to 61 days after treatment a gland was transferred. The original animal was observed during 90 days for relapse and the transfer animal was watched for 90 days for evidence of infection around the site of inoculation. The drugs used were of the German variety dating to before the war, but may be supposed to differ from other brands only in degree of effectiveness.

B. Result.

Of six animals treated in this way, not one gave evidence of infection, even after a single intravenous dose (0.01 gm. per kilo of "606;" 0.015 gm. per kilo of "914"). It is known (Brown and Pearce) that rabbits show clinical relapses after subcurative doses. It may

TABLE IV.

Results of Treatment.

Experiment No.	Rabbit No.	Treatment.	Time after infection.	No. of animal receiving gland.	Time after treatment.	Result of gland transfer.	Remarks.
			days		*days*		
1	38	One dose of neoarsphenamine, 0.015 gm. per kilo.	130	44	28	—*	Latent case. Two large chancres healed before treatment. No relapse within 97 days.
2	39	One dose of arsphenamine, 0.01 gm. per kilo.	130	45	28	—*	Old case still active at time of treatment. No relapse within 97 days.
3	40	Two doses of neoarsphenamine.	125	46	29	—*	Old case, still active at time of treatment. No relapse within 90 days.
4	41	Two doses of arsphenamine.	174	47	29	—*	Old case, still active at time of treatment. No relapse within 90 days.
5	42	Four doses of arsphenamine.	110	48	48	—*	Old case, still active at time of treatment. No relapse within 104 days.
6	43	Four doses of arsphenamine.	125	49	61	—†	Transfer from No. 49 to No. 50 negative after 91 days.

* Observed 90 days.

† Observed 28 days.

be objected that 28 days was too short a time for the spirochetes to recover from the effects of treatment and transfer. It is true that clinical relapses occur in the 1st and 2nd months and 4 weeks seemed a fair time for some of the animals, but two of them were held for 8 and 9 weeks before transfer. This surely gives sufficient time for infection to develop. We do not wish to argue from this experiment that a single full intravenous dose is curative in man any more than we wish to warn against mercurial poisoning from prophylaxis. The rabbit has its own characteristics and responds to drugs in its own way. What we do wish to emphasize is that by the most rigid tests available, syphilis has been abolished by arsphenamine in a mammal. From this it may be concluded that by sufficient treatment the same can be accomplished in man. By cure is meant the death of the last spirochete (17).

IV.

Venereal Spirochetosis of Rabbits.

Noguchi (18) has recently called attention to the occurrence in rabbits of a natural venereal spirochetosis. This disease was previously described by certain European observers, notably Arzt and Kerl (19) and more recently by Schereschewsky (7) and Klarenbeek (20). The work of these and other authors is reviewed by Noguchi. The disease consists of ulcerative papular lesions covered with crusts, principally on the vulva or on the sheath of the penis. Examination of serum expressed from the lesions reveals spirochetes, usually in large numbers, similar, morphologically, to *Treponema pallidum*. Noguchi suggests the name *Treponema cuniculi* for these organisms. The disease is chronic, and persists over an indefinite period of time, with occasional inflammatory exacerbations, during which the area involved is reddened and swollen. These inflammatory exacerbations are easily brought on by manipulation of the parts. The disease occurs under natural conditions and Noguchi found it in five out of fifty animals regarded as normal.

During the course of the present work every possible precaution was taken to prevent any complication of the experiments with this condition. Careful and repeated examination of the stock revealed no lesions. All incoming animals were examined, and they were not

used in experiments until they had been under observation 2 weeks or more. The repeated examination of the animals revealed no lesions resembling those described as due to *Treponema cuniculi*. In order that we might have a close personal experience with the condition and in view of our inability to find it among our animals, we requested Dr. Noguchi to send us one of his animals with lesions of the disease. He kindly sent us a female that had had lesions on the vulva for months. The lesions showed numerous spirochetes with the tendency to form the entangled masses of stellate arrangement noted by Dr. Noguchi. The organisms as a rule seem longer than *pallidum* and somewhat more slender, but in some instances it is impossible morphologically to distinguish the two kinds.

We inoculated the skin of the scrotum of a normal rabbit with serum containing spirochetes from this female, in the same manner in which the animals in the prophylaxis experiments were inoculated. When this animal was examined 5 days later the site of inoculation was slightly reddened and edematous. Examination of serum from the lesion showed an occasional spirochete. Hence the inoculation was positive after an incubation period of only 5 days. The lesion 16 days after inoculation was a slightly raised area about 0.75 cm. in diameter, of much the appearance of an early chancre (Fig. 2). However, it remained stationary, except for becoming slightly more diffuse, for 14 weeks, when it acquired the character of a very superficial non-indurated ulceration. When last observed, 20 weeks after inoculation, the lesion was still present and positive for spirochetes. The persistence of the lesion was in marked contrast to syphilitic lesions which either disappeared completely in a short time, or else went on to the formation of large typical chancres.

A lymph gland was removed from this animal 7 days after inoculation and implanted in the scrotum of a normal animal. A second gland was removed on the 43rd day and transplanted in a second animal. Both these animals receiving gland transplants remained free of any evidence of the disease during the observation period of 90 days. Noguchi was similarly unable to transmit the infection by testicular injection of lymph gland emulsions from ten animals infected with *Treponema cuniculi*. We also transferred a gland from the female sent us by Dr. Noguchi with negative result. Hence,

both in the character and the course of the original lesion and in the failure of gland transfer, there is a marked difference from syphilis.

Noguchi attempted to transmit the disease by mating infected animals with normal animals. He was successful in one instance. Schereschewsky (7), however, was able to transmit the disease sexually with great ease, and, as mentioned above, utilized this method of transmission for testing the value of his prophylactic ointment containing quinine, "Duanti."

The Wassermann reactions of two animals infected with *Treponema cuniculi* were negative, as found by Dr. Noguchi. However, of thirteen of our animals known to be infected with syphilis, only four had positive Wassermann reactions. Hence, the Wassermann reaction seems to be of little aid in differentiating the two conditions.

We do not believe that this disease has been mistaken in the present work. The material for inoculation was invariably obtained from large indurated chancres and the lesions considered syphilitic by us developed at the site of inoculation.

However, lesions due to *Treponema cuniculi* did occur later in two of the rabbits, No. 51 (used in other experiments; Fig. 3) and No. 52 (Fig. 4), one with syphilitic lesions and the other without.

On the 55th day, after inoculation with syphilitic virus, Rabbit 51 had large bilateral chancres. At this time, during the routine examination of the genitalia, it was noted that there were small superficial scaly lesions on the sheath of the penis. Examination of these revealed a few spirochetes. Transfers were made to other rabbits from the chancres and from the lesion of the sheath. The material from the chancre produced a typical large chancre. Serum expressed from the lesion on the penis and subcutaneously injected in the scrotum of a second animal produced an initial lesion after 34 days that was positive for spirochetes. This lesion proceeded to slight superficial ulceration, without induration, and persisted without change until the animal died on the 77th day. Spirochetes were constantly found in it. The difference in these two transfers from the same animal was striking. A transfer from Rabbit 52 to a normal animal also produced a small elevated lesion, proceeding to superficial ulceration, which persisted without showing any induration.

In order to determine whether *Treponema pallidum* would produce a lesion on the sheath of the penis apt to be mistaken for *Treponema cuniculi*, a rabbit (No. 53) was inoculated with syphilitic virus by scarifying the sheath.

41 days after inoculation the mucous membrane of the sheath was swollen and ulcerated at the site of inoculation. Examination for treponemata was positive. The lesion rapidly advanced, and induration of the sheath was marked. To the touch it was hard and cartilaginous. There was no tendency to scaling as in lesions due to *Treponema cuniculi.* The appearance 65 days after inoculation, at which time, however, the lesion had begun to subside, is shown in Fig. 5, for comparison with the *cuniculi* lesion in Fig. 4.

We have, then, a disease of rabbits due to a spirochete often indistinguishable morphologically from the organism of human syphilis. Rabbits infected with it do not have positive Wassermann reactions, while rabbits with human syphilis sometimes have positive reactions. The natural disease takes the form of ulcerated papules in the region of the genitalia. Inoculation of the material into the scrotum may produce lesions somewhat resembling early chancres, as in Dr. Noguchi's rabbit and our rabbit mentioned above. These lesions, however, persist, without either disappearing or advancing (except for superficial ulceration), in contrast to those of syphilis. In syphilis, when the lesion does not go on to the formation of a typical chancre, it immediately disappears, as exemplified in Rabbits 6 (Table I) and 14 (Table III).

Spontaneous lesions of the rabbit disease apparently do not occur on the scrotum, and, when experimentally induced there, differ from the characteristic button-like chancre of the strain of *pallidum* used in the present work. We are of the opinion, therefore, that this natural disease is not a more serious complication in work with lesions of the scrotum and testicle than the disturbance caused by other acid-fast organisms in work on experimental tuberculosis. Experimental work on generalized syphilis, however, and especially on sexual transmission, should be considered with this disease in mind. In its effect on the rabbit, it is more comparable with venereal infection with the organisms of Vincent's angina in man than with the disease due to *Treponema pallidum.*

V.

SUMMARY.

1. By inoculating the scarified surface of both sides of the scrotum of rabbits with suspensions of *Treponema pallidum,* 100 per cent of

infections were obtained on one side or the other. Infection through the unbroken skin could not be produced.

2. By gland transfers from animals with positive local inoculations, 87.5 per cent of takes were produced.

3. These two methods were used to test the prophylactic value of 30 per cent calomel ointment. (*a*) Calomel ointment proved efficacious up to 8 hours after inoculation with syphilis. (*b*) No marked difference appeared between the action of calomel in a base of lanolin and vaseline and in a base of benzoinated lard and wax. (*c*) Death from mercurial poisoning was produced in rabbits by a single application of a large amount of calomel ointment.

4. The method of gland transfers was used to test the sterilizing effect of arsphenamine and neoarsphenamine on old infections in the rabbit. The infection was completely abolished in every instance, whether by one, two, or four intravenous doses.

5. Natural spirochetosis of rabbits need not be a serious complicating factor in work on syphilis in rabbits, for the following reasons. (*a*) In natural spirochetosis, the lesions occur on the penis and not on the scrotum. Gland transfers are negative. (*b*) A scrotal lesion can be produced by inoculation, but it can be distinguished from that of *Treponema pallidum* infection by its course. (*c*) In studies of generalized syphilis supposed to involve the genitalia, and in sexual transmission experiments, *Treponema cuniculi* may be a serious complicating factor.

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EXPLANATION OF PLATE 28.

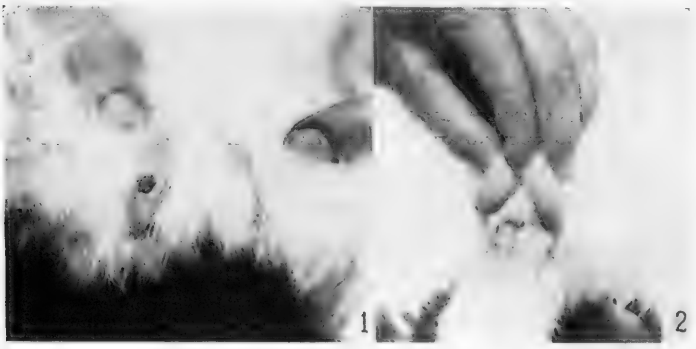
FIG. 1. Rabbit 5; control; 54 days after inoculation. Chancre on the right side. There was no lesion on the left.

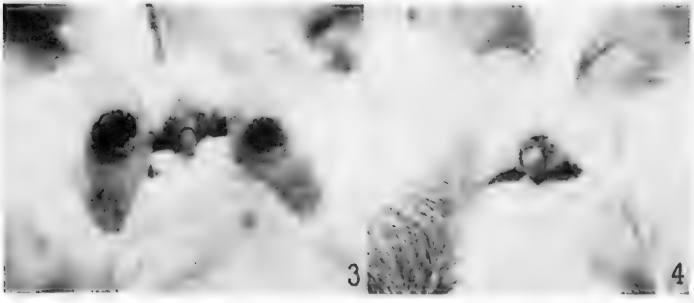
FIG. 2. Rabbit 54. Lesion due to *Treponema cuniculi* on the right side of the scrotum 16 days after inoculation.

FIG. 3. Rabbit 51; 65 days after inoculation. Large bilateral chancres. The sheath of the penis has been drawn back. Lesions, positive for *Treponema cuniculi*, are barely visible in the photograph, on each side of the sheath of the penis just where the mucous membrane joins the skin covered with hair.

FIG. 4. Rabbit 52. Scaly lesions of *Treponema cuniculi* on the sheath of the penis.

FIG. 5. Rabbit 53; 65 days after inoculation of the sheath with *Treponema pallidum*. Chancre of the sheath of the penis.





(Nichols and Walker: Prophylaxis and treatment of syphilis.)

THE MECHANISM OF BACTERIOSTASIS.*

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Plates 29 and 30.

(Received for publication, November 6, 1922.)

The effect of bactericidal agents is often tested by adding these substances to the media on which the organisms are planted, and the assumption is usually made that if the substances, when present in the media, exhibit a selective hostility to bacteria they will exhibit a hostility, selective in the same sense, when added directly to the organisms themselves. This assumption is usually justified by the facts. In the large number of experiments made with gentian violet and allied dyes no single exception to this parallelism between the selective behavior of the dye when present in the media, and its selective behavior when added directly to the bacteria, has ever been encountered; organisms which are "killed" by exposure to the stain never grow when it is present in the media on which they are planted unstained; and organisms which are not affected by exposure to the dye grow readily on media containing it (Fig. 1). We are in the habit, therefore, of reasoning from experiments which test bacteriostasis to conclusions as to bactericidal value, at least as far as selective features are concerned.

Evidence will here be presented to prove that a parallelism of this kind does not always hold, that, on the contrary, certain dyes exhibit one type of selective activity when they are present in the media on which bacteria are grown and a selective activity of the opposite type when the same species of bacteria is stained and then planted on plain agar. Acid fuchsin is a dye which behaves in this way.

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

If *Bacillus prodigiosus* and *Bacillus megatherium* be planted on acid fuchsin agar the former grows well and the latter not at all (Fig. 2, *b*). One would certainly expect from this result that if the two organisms were exposed to acid fuchsin and planted on plain agar the latter would be killed and the former unaffected; but, as a matter of fact, when the experiment is done in this way the opposite effect occurs; *Bacillus prodigiosus* is "killed" and *Bacillus megatherium* unaffected (Fig. 2, *a*).[1] With this dye, therefore, selective bacteriostasis of one type is exhibited when the dye is present in the bacterial environment and selective bacteriostasis of an opposite type when the dye is incorporated in the bacterial bodies. This result is in sharp contrast to the result of similar experiments done with gentian violet, for when this dye is used the selection between Gram-positive and Gram-negative organisms is always in the same sense, whether the dye be added to the media on which the organisms are planted unstained or incorporated in the bacteria before they are planted on plain agar.

It has been abundantly shown in previous publications that organisms exposed to the aniline dyes and apparently killed, may subsequently revive, and what seemed to be death prove to be suspended animation. It is therefore more accurate to avoid the term bactericide in this connection and to discuss the facts in terms of stasis. If the bacteriostasis which a substance exhibits when bacteria are exposed to it (before being planted on plain agar) is described as intrinsic bacteriostasis, and that which a substance exhibits when it is present in the media on which the unstained bacteria are planted, as extrinsic bacteriostasis, then it may be said that the intrinsic selective bacteriostatic features of a substance, far from necessarily running parallel with its extrinsic selective features, may run directly counter to them.

The facts just cited may be demonstrated by experiments in which other substances than acid fuchsin are used. The flavines,[2] for ex-

[1] As stated in a previous publication in which the reverse selective activity of acid fuchsin has been described (Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 1) the effect of this dye is most clearly brought out only when the exposure of organisms to stain is made at a slightly elevated temperature (45°C.).

[2] Neutral proflavine and neutral acriflavine, kindly furnished by the Pharmaceutical Division of the National Aniline and Chemical Company, Inc., New York, were the flavines studied.

ample, exhibit a reverse intrinsic selective bacteriostasis, just as acid fuchsin does;[3] they "kill" *Bacillus pyocyaneus* more readily than they "kill" *Bacillus anthracis* (Fig. 3, *a*; *cf.* with Fig. 1, *a*). If, however, these two organisms are planted, unstained, on agar containing flavine the growth of *Bacillus anthracis* is inhibited, that of *Bacillus pyocyaneus* uninfluenced (Fig. 3, *b*; *cf.* with Fig. 1, *b*); the extrinsic selective bacteriostatic power of these dyes is not reverse but normal.

It appears, indeed, that the growth of Gram-positive organisms may be inhibited by the presence in the media of any one of a number of different substances which have no deterrent effect whatever on the growth of Gram-negatives, and that these substances may be unrelated chemically. The basic triphenylmethanes exhibit this type of extrinsic selective bacteriostasis most sharply. But the acid triphenylmethanes also exhibit it, as do neutral proflavine, neutral acriflavine, Bismarck brown, in strong dilutions, and, according to Kämmerer,[4] mesohematin. With the basic triphenylmethanes, as with gentian violet, extrinsic and intrinsic bacteriostasis run parallel; Gram-positives are inhibited by the dye, whether it be added to the media on which they are grown or added directly to the bacteria before planting them on plain agar, and Gram-negatives are unaffected. This does not occur if the experiments be done with acid fuchsin or the flavines; the predilection of these dyes between Gram-negatives and Gram-positive spore bearers is of one sort when the stain is added to the organisms (intrinsic bacteriostasis) and of the opposite sort when the stain is added to the media on which they are grown (extrinsic bacteriostasis).

This fact was first encountered in comparing the effect of a basic triphenylmethane (magenta) with that of an acid triphenylmethane (acid fuchsin). Since the latter differs from the former only by being

[3] In a previous publication selective bacteriostasis has been described as "normal" when it is similar to that of gentian violet and "reverse" when it is contrary to it (Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 1).

[4] Kämmerer (Kämmerer, H., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 247) has shown that mesohematin ($C_{34}H_{36}O_4N_4FeOH$) and certain metallic compounds of mesoporphyrin, *e.g.* compounds with manganese and magnesium, exhibit the same type of selective activity as gentian violet (normal extrinsic selective bacteriostasis).

sulfonated, it was thought that the H ion concentration of the agar might explain the results, that the sulfonation of the acid fuchsin might be negatived by the agar and that the dye, when added to the media, might be to all intents and purposes changed to a basic dye. The experiments were therefore repeated with agars varying from pH 5 to 10. The results were the same as before. Within the limits of growth for *Bacillus pyocyaneus* and *Bacillus anthracis* (pH 5.4 and 9) the acid fuchsin always exhibited normal extrinsic selective bacteriostasis.

Another suggested explanation for the phenomenon was that the dyes owed their selective power to the selective suppression of some element in the media (salts, growth-accelerating factor, etc.) essential to the growth of Gram-negatives but not essential to that of Gram-positives. But when the experiment was reduced to its lowest terms by repeating it with media consisting of nothing but agar dissolved in distilled water, a medium in which both *Bacillus pyocyaneus* and *Bacillus anthracis* will grow, acid fuchsin, gentian violet, and the flavines still inhibited Gram-positive spore bearers and were without effect on Gram-negatives.

Nothing has been said about the strength of dye used in the experiments. No effort has been made to determine exactly what strength of stain is necessary to inhibit *Bacillus anthracis* while allowing *Bacillus pyocyaneus* to grow, or to kill the latter while sparing the former; this may well be a different strength from the one, for example, which allows *Bacillus typhosus* to grow while inhibiting *Bacillus sublitis*. Such quantitative determinations were unnecessary. Always it was the comparative effect of a given strength of dye on two groups of organisms which was being investigated, not the absolute effect of any given dilution. As long, therefore, as the conditions were kept the same for the two groups of organisms one could draw justifiable conclusions from the results without considering what strength of dye had been used. The method of divided plates made it possible to study extrinsic bacteriostasis (the effect of the dye in media) on the two groups of organisms with complete control and under identical conditions. And in studying intrinsic bacteriostasis (the effect of the stain on the organisms themselves) the two groups of bacteria were always exposed to the same amount of dye, of the same strength, and for the same length of time.

These dyes will kill all organisms if used in sufficiently strong concentrations and will also prevent the growth of all organisms if present in sufficient quantity in the media. On the other hand, if the concentration is weak the dyes may be without effect on either group. Between these two extremes a strength of dye is found which has the selective effects described. As the strength of acid fuchsin and the flavines is increased from the entirely ineffective dilution it is always the Gram-negatives which are killed first, in the intrinsic experiments, and always the Gram-positive spore bearers which are first inhibited in the extrinsic experiments. With gentian violet it is always the Gram-positives which are first affected in both intrinsic and extrinsic experiments.

In experiments with *Bacillus anthracis* and *Bacillus pyocyaneus* it is found that there is a wide range between the strength of dye necessary to kill or inhibit the former and that required to kill or inhibit the latter; and clear-cut results may be readily obtained by making the dilutions colorimetrically with the eye. With *Bacillus anthracis* and *Bacillus typhosus* the range is narrower and it is not easy to obtain such clear-cut results. But whenever there is any predilection, it is always of the kind described, never of the opposite kind.

It is clear that ignorance of the fact that intrinsic and extrinsic bacteriostasis do not always run parallel may lead to erroneous conclusions. Suppose for example that we are testing the effect of acid fuchsin on bacteria by planting them on media containing the dye. We find that the growth of *Bacillus anthracis* is completely inhibited by a strength of dye which has no effect whatever on the growth of *Bacillus coli*. We conclude that acid fuchsin is selectively bactericidal for *Bacillus anthracis* as compared with *Bacillus coli*. If, however, we expose these two organisms to the dye and plant them on plain agar, the reverse proves to be true; *Bacillus anthracis* grows readily, *Bacillus coli* not at all. The action of the flavines is similar.

These substances will kill all organisms if exposure be long enough and to sufficiently strong dilutions; but the Gram-negatives are much more susceptible than Gram-positive spore bearers. Yet when the dyes are added to media on which the organisms are planted unstained, this selective susceptibility is changed; Gram-negatives, like *Bacillus pyocyaneus*, will grow in the presence of considerable amounts of

dye, while spore bearers, like *Bacillus anthracis*, are readily inhibited by small amounts.

There seems little doubt that these facts explain, in part, some of the discrepancies between laboratory experiments and clinical results. The flavines, for example, known on the basis of sound experimental evidence to possess striking bactericidal powers, have been widely used in wounds. The successful results have doubtless been partly due to the power of these dyes to kill organisms with which they come in contact. But since the dyes have often been used only on gauze packed into the wounds, in which case the stain would hardly reach many organisms in strengths sufficient to kill them, the results are probably in many instances due rather to the ability of the dyes to inhibit bacterial growth. Over Gram-negatives, as we have seen, their extrinsic bacteriostatic power is relatively weak; that is to say, when present in the environment but not penetrating the bacteria themselves the flavines allow the Gram-negatives to grow in strengths which will completely suppress Gram-positive spore bearers. Yet when these two groups of organisms are exposed to the dye so that it penetrates their bodies it is the Gram-negatives which are easily "killed" by a strength of dilution which may be without effect on Gram-positive spore bearers. Ignorant of this discrepancy between extrinsic and intrinsic bacteriostasis and familiar with the sharp intrinsic effect of the flavines on the *Bacillus coli* group, observers have noted with surprise the persistence of these organisms in wounds treated with the flavines. In one of the few careful bacteriological studies of wounds made during the war the significant observation is made by Bashford[5] that though in wounds treated with Dakin's solution it is the cocci which disappear last, in wounds treated with flavine the commonest type of organism to outlive others on the wound surface was a Gram-negative bacillus. The same kind of result was obtained in my efforts to sterilize amputation stumps with gentian violet at the Walter Reed Hospital. It was comparatively simple to get rid of the Gram-positive *Bacillus diphtheriæ* but very difficult to abolish the Gram-negative *Bacillus coli*.[6]

[5] Bashford, E. F., Hartley, J. N. J., and Morrison, J. T., *Brit. Med. J.*, 1917, ii, 849.

[6] Churchman, J. W., *J. Am. Med. Assn.*, 1920, lxxiv, 145.

In the studies with gentian violet emphasis has from the first been placed on bacteriostatic and not on bactericidal powers. At least the distinction between the two has been kept constantly in mind and the clinical experiments have been made in an attempt to apply to the treatment of infections the principle of bacteriostasis. Within certain obvious limits this principle is a sound one. The term bacteriostatic is more conservative than bactericidal, and it is the one that should generally be used, for the great majority of experiments in this field, which are supposed to prove that the chemical substance under study has killed the experimental organisms, really prove only that they have been injured or that their growth has been inhibited. Gentian violet, for example, is correctly described as an extrinsic and intrinsic bacteriostat for *Bacillus anthracis*. It prevents the growth of this organism when present in the media on which the plant is made; and when the bacteria are stained with it and planted on plain agar they never reproduce until several days have elapsed, and in the vast majority of experiments do not reproduce at all. That they are not all always killed by staining with gentian violet is proved by the fact that now and then they do reproduce after an interval of quiescence. I have seen stained *Bacillus anthracis*, injected in enormous doses into a mouse, revive after 24 days of complete quiescence and kill the animal, the controls having died in 36 hours.[7] Bactericide would therefore be an incorrect term in this connection.

There is a good deal of reason for supposing that bacteriostats may act by paralyzing the reproductive mechanism without otherwise injuring bacteria (genesistasis). That powerful action on cell processes can be exerted by substances which do not penetrate beyond the surface of the cell is shown by an interesting experiment by Warburg.[8] This observer found that the O consumption of the fertilized eggs of the sea urchin in an artificial sea water is doubled by the addition of 10 cc. of 0.1 N NaOH to 1 liter of sea water, the development being at the same time stopped. If the cells are stained previously with neutral red, which does not affect their development, no change of color takes place when the NaOH is added, showing that the latter has not permeated the cell; whereas with ammonia, to which the cell

[7] Churchman, J. W., *J. Exp. Med.*, 1921, xxxiii, 579.

[8] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

membrane is permeable, the cells become yellow in less than 1 minute. Although the cells are uninjured by the concentration of ammonia used, the O consumption is only increased 10 per cent instead of the 100 per cent when the H ion concentration is changed only at the surface.

There is evidence for the conclusion that gentian violet has an inhibiting effect on the fission of paramecium even in dilutions which do not penetrate the cell or otherwise injure the organism; and in one instance I was able to observe a paramecium, lying in gentian violet, begin its division but never conclude it—a striking example of genesistasis (Fig. 4).[9]

That static effects may be produced without penetration is therefore clear. But it is equally true that penetration of a dye may occur without stasis. In Fig. 5, Tube *a* has been stroked with thirty stained organisms (*Bacillus coli*) picked out individually by the Barber technique, to avoid the chance of having the growth which occurs spring from organisms which had escaped the stain. The luxuriant growth proves that stained organisms can reproduce.

SUMMARY.

1. Between Gram-positive and Gram-negative organisms gentian violet exhibits the same type of selective activity whether the dye be added to the media on which the bacteria are planted unstained (extrinsic bacteriostasis), or the organisms be stained with it before being planted on plain agar (intrinsic bacteriostasis). In both instances the Gram-positives are inhibited and the Gram-negatives unaffected.

2. Between Gram-positive spore-bearing aerobes and the commoner Gram-negative bacteria, acid fuchsin, related sulfonic substances, and the flavines exhibit one type of selective activity when the dye is added to the media (extrinsic bacteriostasis) and the opposite type when it is added directly to the bacteria (intrinsic bacteriostasis). In the former case, the Gram-positive spore bearers are inhibited and the Gram-negatives unaffected; in the latter case the Gram-negatives are inhibited and the Gram-positive spore bearers unaffected.

[9] Busk showed that trypan red had a genesistatic effect on paramecium (Busk, B., cited by Ehrlich, P., in Wolff-Eisner, A., Handbuch der Serumtherapie, Munich, 1910, 316).

3. Selective bacteriostasis is not necessarily conditioned by selective penetrability. Stained organisms may grow, and dyes which do not stain well may inhibit reproduction.

4. There is evidence that the phenomena of bacteriostasis may be due to changes effected by the dye at the surface of the organisms.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1, *a* and *b*. Parallelism of intrinsic and extrinsic bacteriostasis of gentian violet. (*a*) Intrinsic bacteriostasis. *P*, unstained *B. prodigiosus*, for control; *P'*, stained *B. prodigiosus*; *M*, unstained *B. megatherium*, for control; *M'*, stained *B. megatherium*. *B. prodigiosus* has been unaffected, *B. megatherium* inhibited. (*b*) Extrinsic bacteriostasis. Divided gentian violet plate. *B. prodigiosus* has been unaffected, *B. megatherium* inhibited.

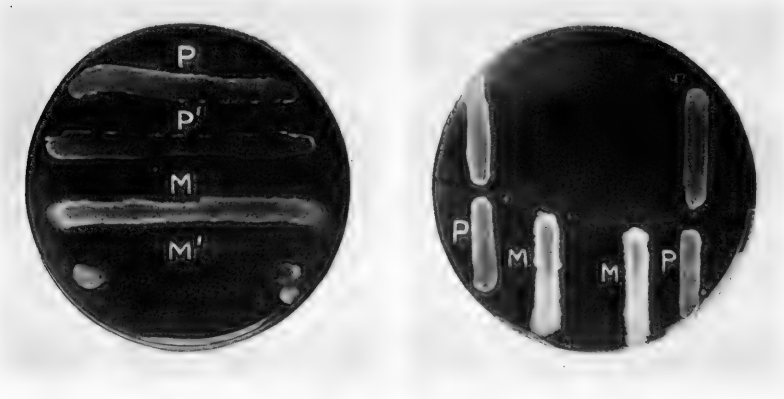
FIG. 2, *a* and *b*. Lack of parallelism of intrinsic and extrinsic bacteriostasis of acid fuchsin. (*a*) Intrinsic bacteriostasis. Lettering the same as in Fig. 1. *B. prodigiosus* has been inhibited, *B. megatherium* unaffected. (*b*) Extrinsic bacteriostasis. *B. prodigiosus* has been unaffected, *B. megatherium* inhibited.

FIG. 3, *a* and *b*. Lack of parallelism of intrinsic and extrinsic bacteriostasis of neutral acriflavine. (*a*) Intrinsic bacteriostasis. *Py*, unstained *B. pyocyaneus*, for control; *Py'*, stained *B. pyocyaneus*; *An*, unstained *B. anthracis*, for control; *An'*, stained *B. anthracis*. *B. pyocyaneus* has been inhibited, *B. anthracis* unaffected. (*b*) Extrinsic bacteriostasis. *B. anthracis* has been inhibited, *B. pyocyaneus* unaffected.

PLATE 30.

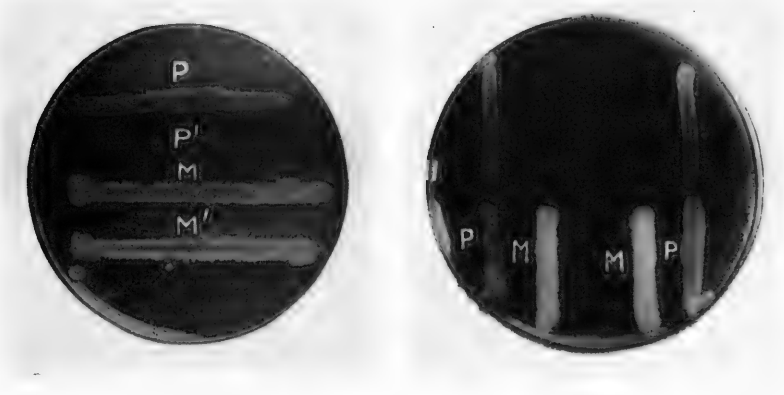
FIG. 4. Paramecium lying in gentian violet solution. Fission has begun but has been arrested and has never proceeded any further. The two nuclei can be plainly seen.

FIG. 5, *a* and *b*. Experiment to show that organisms stained with gentian violet will grow. (*a*) Tube planted with thirty stained individual *B. coli* picked out by the Barber technique; good growth has occurred. (*b*) Tube planted with a similar group of stained *B. subtilis*; no growth has occurred.



a b

FIG. 1.



a b

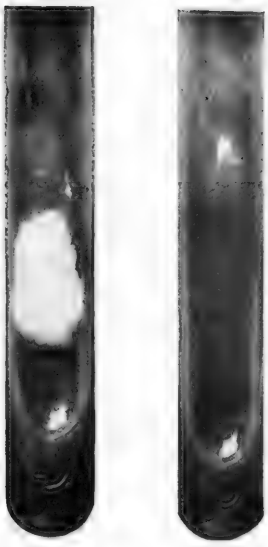
FIG. 2.

a b

FIG. 3.

(Churchman: Mechanism of bacteriostasis.)

FIG. 4.



a b

FIG. 5.

(Churchman: Mechanism of bacteriostasis.)

INFLUENCE OF SODIUM SALICYLATE UPON THE ARTHRITIS OF RABBITS INOCULATED WITH NON-HEMOLYTIC STREPTOCOCCI.

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(Received for publication, December 2, 1922.)

INTRODUCTION.

One of the most striking effects seen in clinical medicine from the administration of drugs is the improvement of patients with acute rheumatic fever following therapeutic doses of salicylic acid or one of its derivatives. So marked is this that the salicylates have gained the reputation of being specifics, and are used to differentiate the arthritis of rheumatic fever from that due to other infections. The reason for this so called specific action—probably better termed characteristic action—has never been satisfactorily explained, although the drug has been used clinically for nearly 50 years. This is not surprising in view of the lack of demonstration of a specific etiologic agent. The opinion seems unanimous that rheumatic fever is an infection; the nature of this infection is still a moot point. Some claim that it is due to a specific microorganism; others, that it is an attenuated pyemia—a specific type of response to a number of different microorganisms of low virulence; still others contend that the arthritis is due to a peculiar type of bacterial sensitization of the joints. These last observers consider the disease an anaphylactic phenomenon.

All of these points of view must be considered in an attempt to explain the characteristic action of the salicylates in rheumatic fever. Pharmacologically, the drugs are antipyretics; but, with the exception of phenylcinchophenic acid, other antipyretics do not affect rheumatic arthritis so favorably. They are analgesics; but the administration of morphine or codeine to patients with rheumatic fever does not relieve the redness, heat, and swelling of the inflamed joints. *In*

vitro they are antiseptics; but the only one which has this action in weak solutions is salicylic acid, and the drug could not exist as such in the body. Such heavy concentrations of the salts are required to kill ordinary pyogenic bacteria that it is impossible to conceive of their existing in bactericidal concentrations in the tissues.

In as far as we are aware, few experiments have been attempted to show a bactericidal action *in vivo*. Hanzlik's (1) complete review of the literature of the salicylates up to 1914 does not mention any studies of this nature. Since then Davis (2) and Fantus, Simmonds, and Moore (3) have studied the effect of salicyl on rabbits inoculated with hemolytic and green streptococci. The former treated rabbits with 0.3 gm. of sodium salicylate subcutaneously each day; three sets of three each were inoculated intravenously with hemolytic streptococci, the fourth set with a streptococcus grown from the joint of a patient with acute rheumatism; "this organism had the general properties of the *Diplococcus rheumaticus* as described by Poynton and Payne." Three controls were inoculated in each experiment. No beneficial effect was observed in the salicylated rabbits; and in general, these animals succumbed more quickly to the hemolytic streptococcus infection than did the controls. All animals developed arthritis of practically the same extent. Although the dose of sodium salicylate employed by Davis was comparable to the largest amount given to patients, the streptococci used in most of his experiments were of such high virulence that a weak antibacterial action of the drug might easily have been masked. In our experience, hemolytic streptococcus infections in rabbits have usually led to purulent arthritis, and often to purulent infiltration of the muscles adjacent to the inflamed joints. These local lesions are more nearly comparable to those seen in patients with osteomyelitis or hemolytic streptococcus infections. No beneficial effect having been obtained from salicylates in these patients, there is no reason to expect one in rabbits.

Fantus, Simmonds, and Moore felt that the dose of salicylate employed by Davis was too small, so they gave rabbits weighing 1 kilo either 0.9 gm. of sodium salicylate, 1 gm. of aspirin, or 1.5 gm. of salophen per day. The rabbits were inoculated intravenously with hemolytic streptococci. The salicylated animals succumbed more rapidly than did the controls. With the exception of aspirin, these huge doses of salicyl did not lead to the death of uninoculated controls. It is probable, however, that the drugs injured the tissues of the rabbits to such an extent that they were less resistant to the infection than were non-salicylated animals. Hanzlik and Karsner (4) have shown that sodium salicylate in doses of from 0.3 to 0.5 gm. per kilo of body weight in dogs and cats leads to severe cloudy swelling of the kidneys, and often to acute tubular nephritis; one rabbit receiving 0.6 gm. per day had marked acute nephritis. When we consider that from 0.15 to 0.2 gm. of salicyl per kilo of body weight is the maximum amount that can be tolerated for 2 or 3 days by most patients without developing severe

albuminuria, it is probable that the animals used by Fantus and his coworkers all had severe renal damage. These experimenters also used hemolytic streptococci for inoculation; hence the criticism made of Davis' experiments is applicable to theirs. In a single experiment of ours, four rabbits were inoculated intravenously with hemolytic streptococci; two received sodium salicylate twice daily by stomach tube. All died within a few days, and all had purulent arthritis. This result is merely confirmatory of the experiments above quoted. It seems evident, therefore, that hemolytic streptococcus infections in rabbits do not furnish suitable conditions for the study of the antibacterial action of the salicylates *in vivo*.

Many workers claim that rheumatic fever is due to infection with non-hemolytic streptococci; and these microorganisms, when recovered from patients with rheumatic fever, have been given the name of *Streptococcus* or *Diplococcus rheumaticus*. The relation of these findings to the etiology of rheumatic fever has been reviewed by one of us (5, 6), and would require too much space to be repeated here. Suffice it to state that they do not fulfill the requirements of Koch's postulates, nor is there any definite immunological proof of their etiological character. In rabbits inoculated with some non-hemolytic streptococci, certain low grade inflammatory lesions are obtained that bear a superficial resemblance to some of the lesions found in patients with rheumatic fever. There does develop a polyarthritis, ranging from a simple increase of clear fluid to a marked increase of practically purulent joint exudate, from which streptococci can be easily recovered in cultures. In joints thus severely inflamed there is usually destruction of the articular surfaces (Cecil (7)). In our experience it has never been possible to recover streptococci from the joints of patients with rheumatic fever; and joint destruction is not a recognized feature of this disease. The endocardial lesions in rabbits resemble more closely the lesions found in patients with non-hemolytic streptococcus endocarditis. The myocardial lesions differ microscopically from the characteristic Aschoff bodies (7–9).

The low grade character of non-hemolytic streptococcus infections, and the fact that following properly graded doses of these organisms rabbits may live for weeks or months, would appear to furnish suitable conditions for the study of the antibacterial action of the salicylates.

EXPERIMENTAL.

For the reasons above stated, it was decided to use non-hemolytic streptococci for inoculation.

In recent years we have collected many cocci of this type from various sources and used them for different purposes; we have found that these strains possess different degrees of virulence and infectivity which are more or less permanent when the stocks are kept in a dried condition after freezing (10). The four strains of streptococci employed in these experiments, Nos. A49, A135, 38D, and Z75, were isolated from blood cultures of patients with rheumatic fever. They have certain cultural differences and immunologically are distinct; they also vary in virulence. In sufficient amounts the first two usually produce arthritis in rabbits; No. 38D sometimes does, but repeated intravenous inoculations of large quantities of No. Z75 are necessary to induce slight arthritis. Before being used in these experiments Strains A49, 38D, and A135 were inoculated repeatedly into the knee joints of rabbits in an attempt to increase their virulence; No. Z75 was first used without preliminary animal passage, but later was passed through several rabbits. The cultures for intravenous inoculation were grown in phosphate buffered broth to which 10 per cent rabbit serum was added; after 18 hours incubation, when the maximum number of actively growing forms was visible microscopically, the cultures were centrifugalized at high speed, the supernatant broth discarded, and the bacterial sediment resuspended in sufficient Ringer's solution to allow 1 or 2 cc. to represent the amount of broth culture indicated in the protocols.

The sodium salicylate was given by stomach tube twice daily in a 2.5 or 5 per cent watery solution. The stomach tube, a No. 10 French catheter, was washed well with water after the drug had passed through, in order to insure that all of the salicylate had been given. To avoid transferring snuffles or other infections from one rabbit to another, a separate tube was used for each animal; this precaution was found advisable as the depressing influence of the streptococcus infection and of large doses of salicylates at times permitted latent rabbit infections to become active.

The rabbits were usually brown, weighing from 1,400 to 2,000 gm.; in each experiment an attempt was made to secure animals of as near the same weight as possible. From an average of several weighings made previously to the inoculation, the dose of salicylate for each animal was computed; subsequently this dose was kept constant, even though the weight of the animals varied. The animals were observed daily for evidence of arthritis. At autopsy, following the inspection of the viscera, the entire skin was removed, and all of the extremities minutely examined; films were made of the fluid from each of the joints whether they appeared abnormal or not. In most of the experiments cultures were made only of fluids from grossly pathological joints; in the fourth experiment cultures were made from all of the joints, and the cocci recovered were identified by

agglutination with specific immune serum. Cultures made from the heart's blood were sterile with one exception. The peritoneum, pleuræ, pericardium, and endocardium were carefully examined for evidence of infection.

In recording the degree of abnormality of the joints, three grades of increased amount of synovial fluid were noted, slight, moderate, and marked; the gross appearance of the fluid varied from clear to thick yellow, with fibrin flakes.

The films of the synovial fluid were stained with Gram's stain; some, in addition, with Giemsa's; the degree of increase of polymorphonuclear leucocytes and mononuclears was roughly estimated and recorded by an increasing number of plus signs. The presence or absence of cocci in the films was also noted. Frequently, irregularly shaped Gram-positive and Gram-negative bodies were seen in the leucocytes, but it was difficult to interpret their nature. At first they were considered definitely as streptococci, but so frequently the fluids in which they were found yielded sterile cultures that doubt was thrown upon this opinion. It is possible that they were killed and phagocytosed bacteria, as fluids yielding positive cultures were seen with cocci varying in appearance from definite chains to bodies similar to those above described. Occasionally, fluids were found that yielded positive cultures, but that showed no microorganisms microscopically.

Experiment 1.—Two groups of six rabbits each were selected. Those of the salicyl group received three doses of sodium salicylate before the first inoculation and twice daily thereafter until death. Rabbits A-76, A-77, and A-78 received 0.2 gm. daily per kilo of body weight; Nos. A-79, A-80, and A-81 received 0.4 gm. for 4 days, when it was evident that they could not tolerate so large a dose and it was reduced to 0.2 gm. On the 1st day all of the rabbits were inoculated intravenously with the sediment from 5 cc. of serum broth Culture A49/8/3,[1] on the 5th day with 10 cc.; and those surviving on the 12th day with 2 cc.

Rabbit A-81 died on the 3rd day; autopsy revealed extensive pneumonia but no arthritis. So short an interval elapsed between the time of inoculation and death that it was considered better to eliminate this animal from the final figures. Because of the poor condition of the other two rabbits receiving the larger doses of salicylate this dosage was reduced one-half; nevertheless, No. A-79 lived only 2 days, and No. A-80 only 4 days longer. Both had arthritis, of the degree shown in Table I. Following the second injection of 10 cc. of culture of streptococci, most of the animals were sick or showed clinical evidence of arthritis; Nos. A-78 and A-79 died the day after the second inoculation. On the 12th day, at the time selected for the third inoculation, only two salicylated animals were living; all of the controls still survived excepting No. A-70, which was killed that morning because of its poor condition. The virulence of Strain A49 made it advisable to reduce the inoculum, so that only 2 cc. were injected. 2 days later another salicylated animal, No. A-77, died; and on the 16th and 17th days the remainder of the animals were killed and autopsied. The positive postmortem findings in the joints are given in Table I.

[1] A49 indicates the strain of streptococcus; /8, eight rabbit passages; /3, third subculture after last animal passage.

TABLE I.

Distribution of Arthritis among the Animals of Experiment 1.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.*	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.†	Character.‡	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.§	Mononuclear cells.§	Bacteria.‖	Culture.
Salicylated rabbits.												
		days										
A-76	3	17	L. S.	0	++	++	0	+	+++	++	0	0
			R. W.	±	++	+±	0	0	+++	++	?	0
			L. K.	±	+	+	0	±	+++	+	?	–
			R. K.	0	0	+	+	0	+++	+	0	–
			L. W.	±	0	+	0	0	+	+	–	–
A-77	3	14	L. S.	0	+++	+++	0	0	+++	++	±	+
			R. S.	0	+++	+++	0	0	+++	++	±	0
			R. A.	±	++	+±	0	+	+++	+++	+	0
			R. E.	0	++	+±	+	0	++	++	±?	0
			L. A.	0	+	+	0	0	+	+	±?	–
A-80	2	9	R. S.	0	+++	+++	0	+	+++	++	±	0
			R. A.	±	+++	+++	+	+	+++	+	0	+
			R. W.	0	+++	+++	+	0	++	++	0	0
			L. H.	0	+	++	0	0	+++	+	±	+
			L. A.	0	+	+±	0	0	++	±	+	0
			R. E.	0	+±	+	0	0	+	+	0	+
			L. S.	0	+	+	0	0	+	+	0	0
			L. E.	0	+	+	0	0	±	0	–	–
			T. A.	0	+++	+++	0	0	+++	++	++	+
A-79	2	6	L. S.	0	++	++	0	+	+++	++	0	0
			L. K.	0	++	++	0	+	+++	++	0	0
			R. W.	0	++	++	0	0	+++	++	0	0
			R. A.	0	++	++	0	0	++	++	0	0
			L. W.	0	+	++	0	0	++	+	0	0
A-78	2	6	R. E.	0	++	+	0	0	++	++	0	0
			R. W.	0	++	+	0	0	+	+	0	0
			L. K.	0	0	+	0	0	+	+	0	–

* S. indicates shoulder; E., elbow; W., wrist; H., hip; K., knee; A., ankle; T.A., tendo Achillis bursa; R., right; L., left.

† 0 indicates no increase; +, slight; ++, moderate; +++, marked increase. In any column – indicates no observation.

‡ + indicates clear; ++, hazy to turbid; +++, frankly purulent.

§ + indicates few cells; ++, moderate number of cells; +++, many cells.

‖ 0 indicates none seen; ±, doubtful; + and ++, distinct.

TABLE I—*Concluded.*

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Control rabbits.												
		days										
A-70	2	12	R. S.	±	++	++	0	0	+++	+	±	0
			L. K.	−	+	+	0	0	+++	+	0	0
			R. S.	−	0	+	0	0	+++	+	0	−
A-71	3	17	R. S.	±	+++	+++	+	+	+++	+	+	0
			L. S.	−	+++	+++	+	+	+++	+	0	0
			R. K.	±	++	++	+	+	++	++	0	0
A-72	3	16	R. S.	?	+++	+++	+	+	+++	0	±	0
			L. S.	0	+++	+++	+	+	+++	0	±	0
			L. W.	±	+++	+++	0	0	+++	+	±	0
			L. K.	±	++	++	0	0	+++	0	±?	0
			L. H.	0	++	++	+	0	++	0	0	0
A-73	3	16	R. S.	±	+++	+++	+	+	+++	+	±	0
			L. W.	0	+	+±	+	0	+++	+	±	0
			L. S.	0	+	+±	0	0	+	+	0	0
			R. K.	0	0	+	0	0	+	±	±	−
			L. K.	0	0	+	0	0	+	+	±	−
			R. A.	0	0	+	0	0	±	0	0	−
A-74	3	16	R. K.	++	+++	+++	+	+	+++	++	±	+
			L. K.	++	+++	+++	+	+	+++	++	±	0
			L. E.	0	+++	+++	0	±	++	+	0	0
			R. S.	−	++	+++	0	+	+++	+	0	0
			L. W.	+	+++	++	0	+	++	+	0	0
			L. S.	0	+	++	0	+	+++	++	±	+
			R. A.	−	+	+±	+	0	++	0	0	−
			R. H.	−	0	+	0	0	+	0	0	−
A-75	3	16	R. K.	+	+++	+++	+	+	+++	++	±	+
			R. W.	0	+++	++	0	0	+++	++	±	0
			R. A.	0	++	++	0	0	+++	+	0	−
			R. S.	0	+	++	0	0	+++	++	+	+
			L. S.	0	++	++	0	0	+++	++	+	0
			R. E.	0	+	++	0	0	+++	++	0	−
			L. H.	±?	+	++	0	0	+++	++	±	0
			L. A.	0	+	+	0	0	+	0	0	0

One striking result is immediately evident. The animals receiving salicylates succumbed much more quickly to the streptococcus infection than did the controls. It is true that three of them at first received much larger doses than are usually administered to patients; the other three, however, took amounts comparable to those used in the clinic; moreover, one of us (11) has shown that 0.2 gm. per kilo of body weight can be safely given to normal rabbits over long periods. In addition to the general depression from the streptococcus infection, there is a well known local injurious effect of these bacteria on the kidneys; salicylates in large doses have also a distinct nephrotoxic action. The summation of these two depressing influences was doubtless an important factor in causing the earlier death of the drug-treated animals. Two controls and one salicylated animal had marked increase of clear peritoneal and pericardial fluids, which yielded negative cultures; one salicylated animal had several subpericardial hemorrhages; one control had subendocardial hemorrhages; one salicylated animal had both subendocardial hemorrhages and small nodules in the pulmonary valve leaflets. There is, therefore, no distinct evidence that the salicylates either prevented or favored the development of cardiac lesions.

TABLE II.

Summary of Distribution of Arthritis of Experiment 1.

Degree of joint involvement.	Salicylated rabbits.						Control rabbits.					
	Survived, 1.		Succumbed, 4.		Total, 5.		Survived, 5.		Succumbed, 1.		Total, 6.	
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
+++	0	0	6	27	6	22	11	37	0	0	11	33
++	2	40	8	36	10	37	14	47	1	33	15	46
+	1	20	7	32	8	30	1	3	1	33	2	6
±	2	40	1	5	3	11	4	13	1	33	5	15
Total	5		22		27		30		3		33	
Average per animal	5		5.5		5.4		6		3		5.5	

An analysis of the grades of arthritis is given in Table II. Four degrees of severity are summarized; severe (+++), with marked

excess of thick, yellow fluid containing many cells; distinct (++), with excess of turbid fluid showing variously increased amounts of cells microscopically; slight (+), with evident excess of clear fluid showing an increased number of cells microscopically; and very slight (±), with normal amount of clear synovial fluid, showing microscopically the presence of abnormal cells. In some of the joints there was noted a slight or moderate increase of clear or slightly turbid fluid, the films of which showed no abnormal cells. In the final summaries it was thought best to disregard these findings, and to consider as diseased only those joints that gave definite microscopic evidence of abnormality.

Individual variation in different animals is to be expected; it is better, therefore, to consider the results in groups of sufficient size to give comparable averages. Five salicylated and six control animals developed arthritis; the average number of abnormal joints in the S[2] group was 5.4, and in the C[3] group 5.5; so that the salicylates did not prevent a bacterial invasion of the joints. A comparison of the degree of arthritis in the two groups is most interesting; it is evident at once that more severely inflamed joints occurred in the C than in the S group. The criticism might be raised that this result would be expected because the earlier death of many of the salicylated animals might not allow sufficient time to elapse for the more severe grades of arthritis to develop. An analysis of the types of arthritis in the salicylated animals dying before the termination of the experiment reveals, however, that two-thirds of the abnormal joints were severely inflamed. The criticism that early death is the important factor cannot, therefore, be applied in this experiment. Another possibility must be seriously considered; *i.e.*, that the salicyl prevented the development of the more severe grades of inflammation in some of the joints.

Experiment 2.—Because of the high proportion of deaths among the salicylated animals in Experiment 1, it was considered advisable to try only a single inoculation with Streptococcus A49. The animals of S group, Nos. A-83, A-84, and A-86, received sodium salicylate, 0.16 gm. per kilo of body weight, daily for 5 days, then 0.08 gm. for 2 days; three doses of the drug were given before the

[2] S indicates salicylated animals.

[3] C indicates control animals.

animals were inoculated. C group consisted of Rabbits A-87, A-88, and A-90. All of the rabbits were injected intravenously with the sediment of 12 cc. of 18 hour serum broth Culture A49/8/5. Rabbit A-88 died within 12 hours, and revealed no gross lesions at autopsy. The rest of the animals survived for 6 days, when they were killed and examined. The salicylated animals all appeared sick the day following inoculation and continued in poor condition until death; the

TABLE III.

Distribution of Arthritis among the Animals of Experiment 2.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Salicylated rabbits.												
		days										
A-83	1	6	L. K.	+	+++	+++	+	++	+++	++	+	+
			L. S.	0	+++	+++	0	0	+++	+++	0	0
			R. S.	0	+++	++	0	0	+	+++	0	0
			L. E.	0	++	++	0	0	+	+++	0	0
			R. A.	+	+	++	+	+	+	++	0	0
			L. W.	0	+	+±	0	0	++	++	0	−
			R. E.	0	±	+	0	0	+	+	0	0
			R .W.	0	0	+	0	+	++	++	0	−
A-84	1	6	R. W.	0	+	+	0	0	+	++	0	−
			L. H.	0	0	+	0	0	+	++	0	−
			R. K.	0	0	+	0	0	+	++	0	−
			L. W.	±?	+	+	0	0	0	++	0	−
			L. E.	0	0	+	0	0	0	++	0	−
			L. A.	0	0	+	0	0	0	++	0	−
A-86	1	6	R. K.	+	+++	+++	+	++	+++	++	+	+
			R. S.	0	+++	+++	0	0	+++	+	+	+
			L. A.	−	++	+±	0	+	+++	+++	0	0
			R. A.	−	±	+±	0	0	+	+++	0	0
			L. W.	−	0	+	0	0	+	+	0	−
			R. E.	−	+	+	0	0	0	+	0	−
			R. W.	−	+	+	0	0	0	+	0	−
			T. A.	−	+++	+++	0	0	+++	+	+	0

TABLE III—*Concluded.*

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Control rabbits.												
A-87	1	6	R. E.	−	++	+++	0	+	+++	++	+	±
			R. H.	?	++	+++	++	++	+++	++	+	+
			L. H.	?	++	++	+	+	+++	++	+	0
			R. K.	+	++	++	0	+	+++	+	0	0
			R. A.	?	+	+±	0	0	+	+++	0	−
			R. S.	0	+	+±	0	0	+	+	0	−
					+++	++	0	0	+++	+	+	0
A-90	1	6	R. E.	+	++	+++	0	0	+++	+	+	+
			L. E.	+	++	+++	0	0	+++	+	+	+
			R. A.	+	++	+++	0	0	+++	+	0	0
			R. K.	+	++	++	0	0	++	+	0	0
			R. H.	?	+	+±	0	0	+	+	0	0

two surviving controls appeared sick on the 3rd and 4th days after inoculation, but improved on the 5th and 6th days. All of the rabbits except No. A-90 had clinical evidence of arthritis on the 4th day and this one showed stiffness in all of its legs on the 5th day.

At autopsy none of the animals had the increase in pericardial and peritoneal fluid that was found in some of the animals in Experiment 1. One salicylated rabbit, No. A-84, had a small yellow nodule 1 mm. in diameter in the posterior leaflet of the mitral valve; blood cultures from another salicylated animal, No. A-86, yielded two colonies of green streptococci. The distribution of inflamed joints is shown in Table III.

A summary of the four types of arthritis is given in Table IV. The number of animals in each group is small; nevertheless, the same general tendency is noted that was found in the first experiment. The average number of inflamed joints in the S group was slightly more than in the C group; all of the arthritis in the control animals

TABLE IV.

Summary of Distribution of Arthritis of Experiment 2.

Degree of joint involvement.	Salicylated rabbits, 3.		Control rabbits, 2.	
	No.	Per cent.	No.	Per cent.
+++	5	23	6	50
++	6	27	6	50
+	5	23		
±	6	27		
Total	22		12	
Average per animal	7.3		6	

was, however, of a severe grade, while half of the abnormal joints in the salicylated animals were mildly inflamed. As all of the animals lived the same length of time following inoculation, the natural inference is that the salicylates prevented the inflammation from becoming severe in some of the infected joints of the S group animals.

Experiment 3.—Because of the severe infection resulting from the inoculation of rabbits with Streptococcus A49, it was decided to employ a less virulent strain, No. 38D. Two groups of six rabbits each were selected; those with even numbers received sodium salicylate, and those with odd numbers served as controls. 0.16 gm. of the drug per kilo of body weight was given daily, divided into two doses; three doses were administered before the first inoculation. On the 1st and 7th days the rabbits were inoculated intravenously with sediment from 5 cc. of broth culture of Streptococcus 38D/4/1, and on the 13th day with 10 cc. All of the rabbits survived longer than the 12th day, so all received a similar amount of inoculum. Clinically, the animals showed only slight symptoms of depression until the morning after the third inoculation, when five of the salicylated animals and one control appeared sick. On the afternoon of that day Rabbit A-62 died shortly after receiving sodium salicylate; the autopsy indicated that the solution had been injected into the trachea. No. A-56 died on the night of the 14th day; No. A-61 on the 17th day; and No. A-60 on the 20th day. It is fair to attribute the death of only the last three animals to the inoculum. The surviving animals were killed and examined on the 20th and 21st days. At autopsy a slight increase in peritoneal fluid was found in only one of the controls, No. A-57. Four of the salicylated rabbits, on the other hand, had a definite increase in peritoneal fluid, and two of these had a distinct increase of pericardial fluid. Cultures of these clear fluids were sterile; and as the other tissues of these rabbits appeared pale and edematous it was thought that the increase in serous fluid was a part of a general edema. This, occurring only in the salicylated animals, was probably a result of the combined toxic effect of the salicylates and streptococci on the kidneys of the animals. Rabbits A-52 and A-54 had, in addition, small sub-

endocardial hemorrhages into the leaflets of the tricuspid valves. The findings in the abnormal joints are given in Table V. It will be noted that the number of joints involved varied considerably in different rabbits; No. A-52 had no definite arthritis; No. A-54 had only one joint definitely involved, although it had in five other joints a distinct increase of clear fluid containing no cells. As this

TABLE V.

Distribution of Arthritis among the Animals of Experiment 3.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Salicylated rabbits.												
		days										
A-52	3	20	R. H.	0	0	+	0	0	0	0	0	0
A-54	3	21	L. H.	0	0	+	0	0	+	±	0	–
A-58	3	21	R. W.	0	++	++	0	0	+++	++	+	0
			R. E.	0	++	+	+	0	+	++	0	0
			L. A.	0	++	+	0	0	+	++	±	0
			R. A.	±	+	+	0	0	+	++	0	–
			R. K.	±	0	+	0	0	+	++	±	–
A-60	3	19	L. A.	+	+	++	0	0	+	+	0	–
			R. A.	+	±	+	0	0	+	+	0	–
			R. H.	±?	+	+	0	0	+	+	+	–
			R. W.	±?	0	+	0	0	0	++	0	–
			L. E.	0	0	+	0	0	0	++	0	–
A-62	3	14	R. S.	0	+	++	0	0	+++	++	±	0
			L. S.	0	±	+±	0	0	+++	++	±	0
			R. A.	±	+	++	0	0	+	+	0	0
			R. K.	0	0	+	0	0	+	++	0	0
			L. K.	0	0	+	0	0	+	++	0	0
			L. A.	0	0	+	0	0	+	+	0	0
A-56	3	14	L. W.	±?	+	+	0	0	+	+	0	0
			R. K.	±?	0	+	+	0	+	+	0	–
			R. W.	±?	0	+	0	0	+	+	0	–
			R. E.	0	0	+	0	0	+	+	0	–
			R. H.	0	0	+	0	0	±	±	0	–

TABLE V—*Concluded.*

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			Culture.
					Joint fluid.		Tissue.		Joint fluid.		Bacteria.	
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.		
Control rabbits.												
A-53	3	20	R. H.	0	0	+	0	0	+	+	0	–
A-55	3	21	L. S.	0	+	++	0	0	+	++	0	–
			R. S.	0	0	+	0	0	0	++	0	–
			L. E.	0	0	+	0	0	0	++	0	–
A-57	3	20	R. H.	0	+	+±	0	0	+++	++	±	–
			R. W.	±	±	+	0	0	±	+	0	–
			L. A.	0	0	+	0	0	±	+	0	–
A-59	3	21	L. W.	+	+++	++	0	0	+++	++	±	0
			R. S.	0	+++	++	0	0	+++	++	±	+
			R. A.	0	±	+	0	0	+	+	–	–
			L. S.	0	±	+	0	0	0	±	0	–
A-65	3	21	L. S.	0	+	++	0	0	+++	++	±	0
			L. A.	0	+	+±	0	0	+++	++	±	–
			R. W.	±	+	+±	0	0	+	+	0	–
			R. K.	0	+	+±	0	0	+	+	0	–
			R. S.	0	+	++	0	0	+	++	0	0
			L. K.	0	±	+	0	0	+	+	0	–
			L. H.	0	0	+	0	+	+	+	0	–
A-61	3	17	R. K.	0	+	+±	0	+	++	++	+	–
			L. K.	0	+	++	0	0	++	++	+	–
			R. A.	0	+	+±	0	0	++	++	0	–
			L. A.	0	+	+±	0	0	++	++	0	–
			L. E.	0	+	+±	0	0	+	+	±	0
			L. S.	0	+	+±	0	0	+	0	0	–
			L. W.	0	+	+±	0	0	0	+	0	0

animal had so much edema it was thought that this increased fluid was simply part of the general anasarca. No. A-53 had only microscopic evidence of inflammation in one joint; and No. A-55 only slight signs of inflammation in three joints. Thus, the four animals showing slight or no evidence of arthritis are equally divided between the two groups.

The summary of the distribution of the various grades of arthritis in the two groups (Table VI) indicates that Streptococcus 38D was definitely less irritating in the joints than Streptococcus A49, as none of the animals had purulent synovial fluid. There were six rabbits in each group; Group S had an average of 3.7 abnormal joints; Group C, 4.2. The total number of diseased joints in each group approximate each other closely enough to make comparison easy. The ratio of distribution of the various degrees of intensity in the two groups of animals shows that nearly three times as many severely inflamed joints occurred in the control animals. This ratio is almost reversed under the heading "slight." Here, the occurrence of milder

TABLE VI.

Summary of Distribution of Arthritis of Experiment 3.

Degree of joint involvement.	Salicylated rabbits.						Control rabbits.					
	Survived, 3.		Succumbed, 3.		Total, 5.		Survived, 5.		Succumbed, 1.		Total, 6.	
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
++	1	17	4	25	5	23	9	50	6	86	15	60
+	3	50	3	19	6	27	4	22	1	14	5	20
±	2	33	9	56	11	50	5	28			5	20
Total	6		16		22		18		7		25	
Average per animal	2		5.3		3.7		3.6		2.3		4.2	

arthritis cannot be attributed to the early death of half of the salicylated animals, because in both groups the animals dying earlier had more severe arthritis than did the rabbits living the full period.

It has been claimed by Faber (12) and Herry (13) that the localization of streptococci in any particular joint of a rabbit inoculated intravenously is favored by previous sensitization of that joint by intraarticular injection of homologous killed streptococci; Faber used killed cultures and Herry clear extract of streptococci. Both claim that the arthritis in patients with rheumatic fever is explained by their experimental results. We thought that if their experiments could be repeated, the influence of the salicylates might be more accurately studied than in animals in which the development of

arthritis was more or less fortuitous. The knee joints of all of the rabbits used in the next three experiments were, therefore, injected with small amounts of killed cultures of different strains of streptococci 4 or 5 weeks previous to the intravenous inoculation. During the intervening period the injected joints were inspected and measured frequently, but the animals were subjected to no further experimental manipulation until the knees had returned to their former size or were no longer undergoing change. The signs of inflammation usually disappeared after 2 weeks; occasionally there was slight permanent thickening of the joint, and in a few instances there was slight atrophy. As the details of our joint sensitization experiments are given in another communication, only the general results are tabulated here and discussed in so far as they have a bearing on the subject at hand.

Experiment 4.—The knee joints of six rabbits were injected as follows: right knee, with the sediment of 0.5 cc. of Culture 38D/3/4 in 0.5 cc. of normal salt solution; left knee, with a similar amount of Culture A49/8/4 prepared in the same way. The saline suspensions were heated at 56°C. for ½ hour, then plated to prove their sterility. All of the knees showed a definite inflammatory reaction after being injected. 27 days later the three rabbits, Nos. K-23, K-25, and K-28, were given sodium salicylate, 0.2 gm. per kilo of body weight daily, and continued on this treatment until the termination of the experiment. On the 28th day all of the rabbits were inoculated intravenously with the sediment of 9.3 cc. of Culture 38D/4/2, and subsequently observed daily until the termination of the experiment, when they were killed and examined as usual; Nos. K-23 and K-24 were sacrificed on the 8th day, Nos. K-25 and K-26 on the 9th day, and Nos. K-22 and K-28 on the 10th day following intravenous inoculation. At autopsy, cultures were made of all of the joints by mixing one loopful of synovial fluid with freshly prepared dextrose blood agar; plates were poured and incubated 3 days. All colonies resembling those produced by streptococci were picked and planted into serum broth; after microscopic examination this culture was tested with immune rabbit serum that agglutinated Streptococcus 38D. Thus, all of the streptococci isolated from the joints were identified as the same strain that was inoculated intravenously, or else considered as contaminations picked up at the autopsy. The result of gross and microscopic examination of the joint fluid, as well as of the bacteriological examination, is given in Table VII.

Relatively fewer joints were involved in this series of rabbits, doubtless due to the fact that the streptococci were less virulent than those used in the first two experiments, and fewer inoculations were made than in the third. Another point must be mentioned in passing,

TABLE VII.

Distribution of Arthritis among the Animals of Experiment 4.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			Culture.
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	
Salicylated rabbits.												
		days										
K-23	1	8	R. S.	0	+++	+++	+	+	+++	+	+	0
			R. K.	+	+++	+++	++	+	+++	+	+?	+
			R. W.	+	++	+++	?	+	+++	+	+?	0
			L. A.	0	0	+	0	0	0	0	0	+
K-25	1	9	L. S.	0	+	+	0	0	±	+	0	0
			R. H.	0	±	+	−	−	±	+	0	0
			L. K.	0	±	+	0	0	±	+	0	0
			R. E.	0	0	+	0	0	0	+	0	0
K-28	1	10	R. A.	0	+	++	0	0	+++	+	+?	0
Control rabbits.												
K-22	1	10	R. E.	0	+++	+++	0	+	+++	++	+	0
			L. E.	0	±	+±	0	0	+	+	0	0
			R. A.	0	±	+	−	−	0	+	0	0
			R. K.	0	0	+	−	−	+	+	0	0
K-24	1	8	L. K.	+	+++	+++	+	+	+++	++	+?	0
			R. K.	−	++	+	0	0	+++	+	+?	0
			L. S.	−	0	+	0	0	++	+	0	0
K-26	1	9	R. W.	0	++	++	0	0	+	++	0	0
			L. W.	0	±	+	0	0	0	+	0	0

that to the preliminary injection of the knees with killed streptococci cannot be attributed the localization of the arthritis in those joints. In the six rabbits arthritis was found in five knees; but in only three animals was the right knee—the one previously injected with Streptococcus 38D—involved; in two rabbits the left knee, previously

injected with Streptococcus A49, was intensely inflamed. In addition, thirteen other joints in the entire series of animals showed evidence of invasion with streptococci; as these joints had not been previously injured in any manner, it seems highly improbable that the localization of arthritis in any of the joints was due to a preliminary sensitization.

It was necessary to include in Table VII one joint that did not show any microscopic evidence of inflammation because from the left ankle of Rabbit K-23 a pure culture of Streptococcus 38D was obtained. This is probably an instance in which the joint had just been invaded by the bacteria, and sufficient time had not elapsed for a cellular

TABLE VIII.

Summary of Distribution of Arthritis of Experiment 4.

Degree of joint involvement.	Salicylated rabbits, 3.		Control rabbits, 3.	
	No.	Per cent.	No.	Per cent.
+++	3	33	2	22
++	1	11	3	33
+	3	33	2	22
±	2	22	2	22
Total	9		9	
Average per animal	3		3	

reaction to be incited; it is the only example of this kind encountered during the course of these experiments.

The summary of the distribution of the different degrees of inflammation in the two groups of animals (Table VIII) shows that the same number of joints was invaded in each group. Owing to the small number of joints involved no very striking difference is seen; one more severely inflamed joint was found in the S than in the C group. If, on the other hand, *marked* and *severe* are grouped together and the two degrees of *slight* similarly treated, the ratio between S and C in the first is 4 to 5, and in the second, 5 to 4. Considered alone, this experiment furnishes no very conclusive evidence of an inhibitory influence of salicylates, but when correlated with the others it is confirmatory in nature.

Experiment 5.—This experiment was carried out in essentially the same manner as the last. The knee joints of eight rabbits were injected with killed streptococci suspended in 0.5 cc. of normal saline; the right knees of Rabbits K-42, K-43, K-50, and K-51 with 0.5 cc. of Streptococcus Z75; the right knees of Nos. K-46, K-47, K-54, and K-55 with 2 cc. of the same streptococcus; the left knees of the first four rabbits with 2 cc. of Streptococcus A135, and the corresponding knees of the last four with 0.5 cc. of Strain A135. The animals were observed for 34 to 36 days, at the end of which time the joints had returned to their previous condition, and the knee joints of controls similarly injected appeared normal upon postmortem examination. Rabbits K-50, K-51, K-54, and K-55 were then given

TABLE IX.

Distribution of Arthritis among the Animals of Experiment 5.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Salicylated rabbits.												
		days										
K-50	1	8	R. S.	0	++	++	0	0	0	++	−	−
K-51	1	9	L. K.	+	+++	+++	+	+	+++	+	−	−
			R. S.	0	+++	+++	+	+	+++	++	−	−
			L. S.	0	+++	+++	+	+	+++	++	−	−
			L. E.	±	+	++	0	0	++	++	−	−
K-55	1	1	R. W.	0	+++	++	−	−	++	++	−	0
			R. S.	0	++	++	−	−	++	++	−	−
			L. E.	0	+	++	−	−	+	+++	−	+
			L. S.	0	0	+	−	−	++	++	−	−
			L. W.	0	+	+	−	−	+	+	−	−
			R. A.	0	0	+	−	−	+	++	−	−
			L. A.	0	0	+	−	−	+	+	−	−
K-54	1	1	R. K.	0	++	+	0	+	0	++	−	+
			L. K.	0	++	+	0	0	0	++	−	0
			L. W.	0	+	+	0	0	0	+	−	−
			L. H.	0	0	+	0	0	0	++	−	−

TABLE IX—*Concluded.*

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Control rabbits.												
K-42	1	8	R. A.	0	++	+++	0	0	+++	+	±?	0
			L. E.	0	++	++	0	0	+++	±	±?	−
			L. S.	0	+	++	0	0	+++	+	±?	0
			R. E.	0	+	+±	0	0	+++	+	±?	−
			L. H.	0	++	+±	0	0	++	++	0	−
			R. W.	0	+	+	0	0	+	++	0	−
			R. H.	0	0	+	0	0	++	++	0	−
			L. K.	0	0	+	0	0	+	+	0	0
			L. W.	0	0	+	0	0	±	+	0	−
K-43	1	9	L. K.	+	+++	+++	+	+	+++	+	±?	0
			L. S.	+	+++	+++	+	+	+++	+	+	−
			L. A.	0	++	+++	0	0	+++	+	0	0
			R. A.	0	+	++	0	0	+	+	0	−
			R. E.	0	±	+±	0	0	+	+	0	−
K-46	1	8	R. K.	+	+++	+++	+	+	+++	+	+?	0
			R. E.	0	++	+++	0	0	+++	+	+?	+
			R. S.	0	++	+++	0	0	+++	++	+?	+
			L. S.	0	+	++	0	0	+++	++	+?	−
			L. H.	0	+	+±	0	0	+	++	0	−
			L. A.	0	±	+±	0	0	+	0	0	−
			L. K.	0	±	+	0	0	+	0	0	−
			L. W.	−	+	+	0	0	0	+	0	−
K-47	1	9	L. K.	++	+++	+++	+	+	+++	++	+?	+
			L. S.	0	+++	+++	+	+	+++	+	+?	+
			L. W.	0	++	+++	0	0	+++	+	?	0
			R. E.	0	++	+++	+	+	+++	+	?	0
			R. K.	0	+	++	0	0	++	+	0	−
			L. E.	0	++	++	0	0	+	++	0	−
			R. H.	0	0	+	0	0	+	+	0	−

sodium salicylate in the usual manner, 0.2 gm. per kilo of body weight daily until death; three doses were administered before the animals were inoculated. All of the rabbits were then injected intravenously with the sediment from 30 cc. of culture, Streptococcus A135/2/2. Rabbit K-54 died after 20 hours, and No. K-55 after 24 hours. The remainder of the animals surviving the inoculation were observed for 8 or 9 days, when they were sacrificed and examined in the usual manner. No gross visceral lesions were found in any of them. The distribution of arthritis is shown in Table IX.

Although five of the eight rabbits had lesions in the left knees (the joints previously injected with Streptococcus A135), in the entire group of animals, there were forty other inflamed joints, including three right knees previously injected with Strain Z75. In the consideration of the effects of salicylates it is, therefore, only fair to eliminate the question of joint sensitization. The two salicylated rabbits surviving the same length of time as the controls had distinctly fewer pathological joints than did the controls; only five abnormal joints were found in these two animals—an average of 2.5 each—compared with an average of nearly eight per animal in the controls (Table X). It is rather striking that four abnormal joints were severely inflamed; the possibility is, therefore, suggested that if other joints of these two animals were invaded the pathological process was completely inhibited. The other two salicylated animals dying the day following inoculation showed evidence of invasion of more joints; the degree of inflammation was distinctly less than in the animals living 8 days; but this would be expected, because there was insufficient time for the development of a severe arthritis.

TABLE X.

Summary of Distribution of Arthritis of Experiment 5.

Degree of joint involvement.	Salicylated rabbits.						Control rabbits.	
	Survived, 2.		Succumbed, 2.		Total, 4.		Survived, 4.	
	No.	Percent.	No.	Percent.	No.	Percent.	No.	Percent.
+++	3	60	0	0	3	19	10	35
++	2	40	3	27	5	31	12	41
+			5	46	5	31	3	10
±			3	27	3	19	4	14
Total.....	5		11		16		29	
Average per animal.....	2.5		5.5		4		7.2	

It is hardly possible to compare the results in the S and C groups, as has been done in other experiments, because of the early death of two of the salicylated rabbits. While the previous administration of the salicylates may have been a factor in causing the death of these two animals, it seems probable from the total summary column that fewer joints were involved in the salicylated animals than in the controls, as the ratio of S to C is nearly 1 to 2. This result may have occurred instead of the usual shifting of the degree of inflammation from severe to slight.

Experiment 6.—This experiment, in some ways, is a duplicate of the previous one. The right knees of eight rabbits were injected with killed Streptococcus Z75, and the left with No. A135; after 34 to 36 days one-half of these rabbits, Nos. K-48, K-49, K-52, and K-53, were treated with sodium salicylate, 0.2 gm. per kilo of body weight daily; the drug was continued for 19 days. After these rabbits had received three doses of salicylate, all of the animals were injected intravenously with the sediment from 50 cc. of broth Culture Z75/0/11. As no effect was observed from this inoculation, after 7 days all received a similar amount of Strain Z75/1/2; 9 days later a third inoculation of the same amount of Strain Z75/3/2 was made. 19 days after the first inoculation all of the animals were sacrificed and examined; none showed any visceral lesions; the small amount of arthritis in the entire group is indicated in Table XI.

It is evident at once that the previous inoculation did not predispose the knees to arthritis as none of these joints contained abnormal fluid. The relatively low virulence of Strain Z75 is indicated by the fact that although each rabbit received a total of 150 cc. of culture within a period of 16 days, they showed little, if any, systemic disturbance; and at autopsy only a few joints were slightly inflamed. In Table XI, the slightest degree of abnormality is indicated. Two of the salicylated animals, Nos. K-49 and K-53, had no evidence of arthritis at all; and No. K-48 had slightly increased clear, apparently normal, fluid in the right elbow; so that in only one salicylated animal, No. K-52, was there a distinct increase of hazy fluid showing simply an increased number of mononuclear cells microscopically. Three of the control animals had a distinct increase of slightly turbid fluid containing abnormal cells, although in only two joints were polymorphonuclear leucocytes found. In the fourth control, No. K-41, there was an increased amount of normal fluid. Judged by the

standards used in the previous experiments, it would be considered that in only one salicylated animal and in three controls was there arthritis. With so few joints involved it is superfluous to summarize the results in a table. The same tendency noted in the other experiments for the salicylated animals to have less severe arthritis than

TABLE XI.

Distribution of Arthritis among the Animals of Experiment 6.

Rabbit No.	No. of inoculations.	Length of time rabbit lived after inoculation.	Joint.	Clinical arthritis.	Gross examination.				Microscopic examination.			
					Joint fluid.		Tissue.		Joint fluid.			
					Amount.	Character.	Periarticular changes.	Synovial membrane.	Polymorphonuclear cells.	Mononuclear cells.	Bacteria.	Culture.
Salicylated rabbits.												
		days										
K-48	3	19	R. E.	0	++	+	0	0	0	0	0	0
K-52	3	19	L. E.	0	++	+±	0	0	0	±	0	–
K-49	3	19	*	0	0	+	0	0	0	0	0	–
K-53	3	19	*	0	0	+	0	0	0	0	0	–
Control rabbits.												
K-40	3	19	R. W.	0	++	++	0	0	+++	+	+?	0
K-41	3	19	R. S.	0	+	+	0	0	0	0	0	–
K-44	3	19	R. E.	0	++	+	0	0	0	+	0	–
			L. E.	–	++	+±	0	0	0	±	0	–
K-45	3	19	L. E.	0	+	++	–	–	+++	++	–	–

* No abnormal joints.

the controls is evident here. Considered from the point of view of the prevention of the development of arthritis, this experiment is more striking than any of the others. This may be due to the fact that the inoculated streptococci possessed such low invasive properties that the slightest antibacterial action of the drug was evidenced at once by the absence of local lesions.

DISCUSSION.

In considering the general results of the experiments just detailed, the figures may be analyzed in a number of different ways. It is possible that comparing percentage figures may indicate one tendency, while contrasting absolute or total figures may point in a different direction. This is especially true when the total figures in the two groups vary considerably. If, on the other hand, the same tendency is found in each individual experiment and in summaries collated in both ways, it is safe to conclude that there has been a definite influence of the drug on the course of the particular type of infection under consideration.

Table XII is a summary of all of the experiments; it shows the total number of inflamed joints in each category of the various degrees of severity, with the animals divided into those living the full experimental period and those dying earlier; the percentage distribution of the four different degrees of arthritis is also given.

The first striking point is the influence of the drug on the course of the general infection. In each group there were twenty-five animals; nine (36 per cent) of the salicylated rabbits died, but only two (8 per cent) of the controls succumbed before the termination of the experiments. There was a higher mortality among the animals inoculated with the more virulent streptococci. This indicates that sodium salicylate in the doses used had a tendency to depress the general resistance of the animals, a point of considerable interest when contrasted with the apparent influence on the local infection.

The effect of earlier death upon the type of arthritis has already been discussed in the individual experiments; a better idea of this can probably be gained by comparing all of the salicylated animals. The two rabbits of Experiment 5 dying within 24 hours had 70 per cent slight arthritis; the seven rabbits in Experiments 1 and 3 had, on the other hand, only 52 per cent of slight arthritis compared with 50 per cent for the sixteen animals living the full time. The animals dying earlier had, however, 5 per cent more arthritis classified under the heading "marked" than did the surviving animals. In general, the inclusion of the former group in the total of the salicylated animals shifts the percentage figures more from the heading "marked" to "distinct." As half of the inflamed joints in the salicylated animals

TABLE XII.

Summary of All the Experiments Showing the Distribution of the Different Grades of Arthritis.

Salicylated animals	Animals that survived the experimental period.								Animals that succumbed to the infection.					Grand total.	Per cent.
Experiment No.	1	2	3	4	5	6	Total.	Per cent.	1	3	5	Total.	Per cent.		
No. of animals	1	3	3	3	2	4	16		4	3	2	9		25	
Length of time animals lived after inoculation, *days*	17	6	21	10	9	19			6, 6, 9, 14	14, 14, 19	1, 1				
No. of inoculations	3	1	3	1	1	3			2, 2, 2, 3	3	1				
" " joints showing respective degrees of arthritis... +++	0	5	0	3	3	0	11	**23**	6	0	0	6	**12**	17	**17**
" " joints showing respective degrees of arthritis... ++	2	6	1	1	2	1	13	**27**	8	4	3	15	**31**	28	**29**
" " joints showing respective degrees of arthritis... +	1	5	3	3	0	0	12	**25**	7	3	5	15	**31**	27	**28**
" " joints showing respective degrees of arthritis... ±	2	6	2	2	0	0	12	**25**	1	9	3	13	**26**	25	**26**
Total No. of joints							48					49		97	

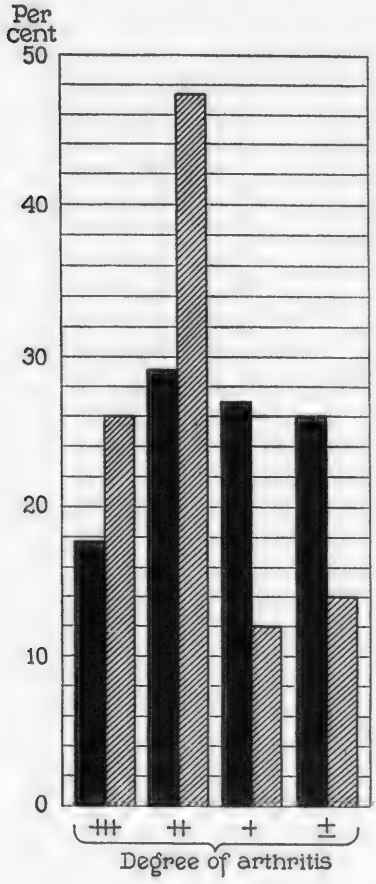
Control animals	Animals that survived the experimental period.								Animals that succumbed to the infection.					Grand total.	Per cent.
No. of animals	5	2	5	3	4	4	23		1	1		2		25	
Length of time animals lived after inoculation, *days*	17	6	21	10	9	19			12	17					
No. of joints showing respective degrees of arthritis... +++	11	6	0	2	10	0	29	**28**	0	0		0		29	**26**
No. of joints showing respective degrees of arthritis... ++	14	6	9	3	12	2	46	**45**	1	6		7	**70**	53	**47**
No. of joints showing respective degrees of arthritis... +	1	0	4	2	3	2	12	**12**	1	1		2	**20**	14	**13**
No. of joints showing respective degrees of arthritis... ±	4	0	5	2	4	0	15	**15**	1	0		1	**10**	16	**14**
Total No. of joints							102					10		112	

occurred in the nine rabbits dying earlier, it seems that the depressing effect of the salicylate upon the general resistance of these animals practically overcame the local influence of the drug; hence in subsequent comparisons we shall consider only two groups, salicylated and controls.

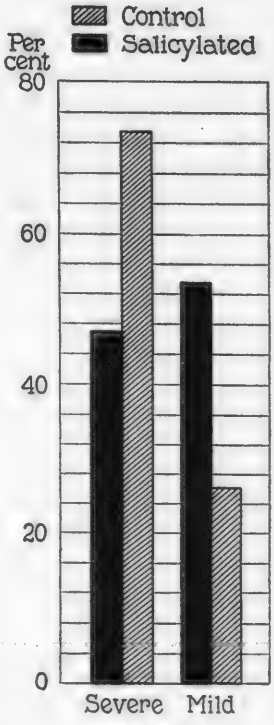
The total number of inflamed joints in the former group was 97, and in the latter, 112. It seems possible, therefore, that the salicylate may have prevented the development of a certain amount of arthritis; this appears more evident in the animals inoculated with the less virulent organisms. To be perfectly certain about this point, however, a much larger number of animals should be studied, the amount and virulence of the inoculum being varied and the dose of salicylate arranged so that few rabbits succumb to the general infection. The percentage distribution of the various degrees of intensity among the total inflamed joints of the two groups is indicated in the appropriate columns and shown in graphic form in Text-fig. 1. Here is brought out the fact that the salicylated animals had a smaller proportion of arthritis classed as marked and distinct and a higher proportion of slightly inflamed joints. This is even more forcibly brought out in Text-fig. 2, in which only two headings, severe and mild, are used, the first including the *marked* and *distinct*, and the second the *slight* and *very slight*.

Because 4 per cent more joints were affected among the controls than among the salicylated animals it was thought advisable to analyze the figures from the standpoint of the relation between the total number of joints in which it was possible for infection to occur and the actual number found diseased in each group of animals. As only the joints of the four extremities were examined, it may be considered that for the purpose of analysis each rabbit had twelve joints. A summary of the findings in each individual experiment, including only two classes of arthritis, severe and mild, is shown graphically in Text-figs. 3 and 4. Here the order of the experiments has been rearranged so that the most severe infection is indicated on the left and the mildest on the right. Text-fig. 3 shows the percentage of inflamed joints considered from the point of view of the entire number of joints capable of being infected in each group. This mode of treating the figures was rendered necessary by the different number

of animals in the S and C groups in Experiments 1 and 2. The proportion of diseased joints was higher in the C group in four experiments, the same in one, and less in one (Experiment 2). Text-fig.



TEXT-FIG. 1.

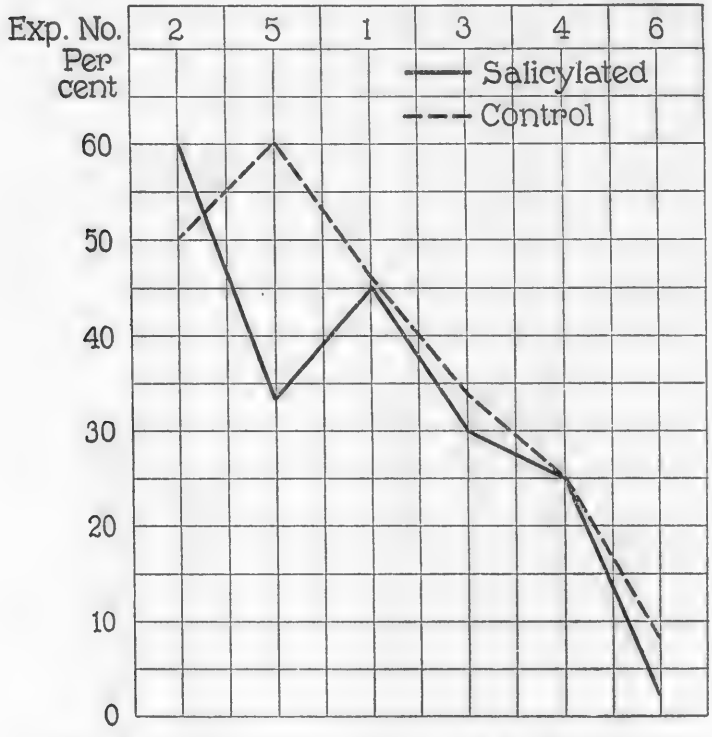


TEXT-FIG. 2.

TEXT-FIG. 1. Percentage distribution of the four grades of arthritis among all of the salicylated and control rabbits.

TEXT-FIG. 2. Percentage distribution of severe and mild arthritis among all of the salicylated and control rabbits.

4 indicates that there was always a higher proportion of severe arthritis among the controls, and, with the exception of Experiment 6, a greater percentage of mild arthritis among the salicylated rabbits. The wide interval between the S and C curves in Experiment 5 is due

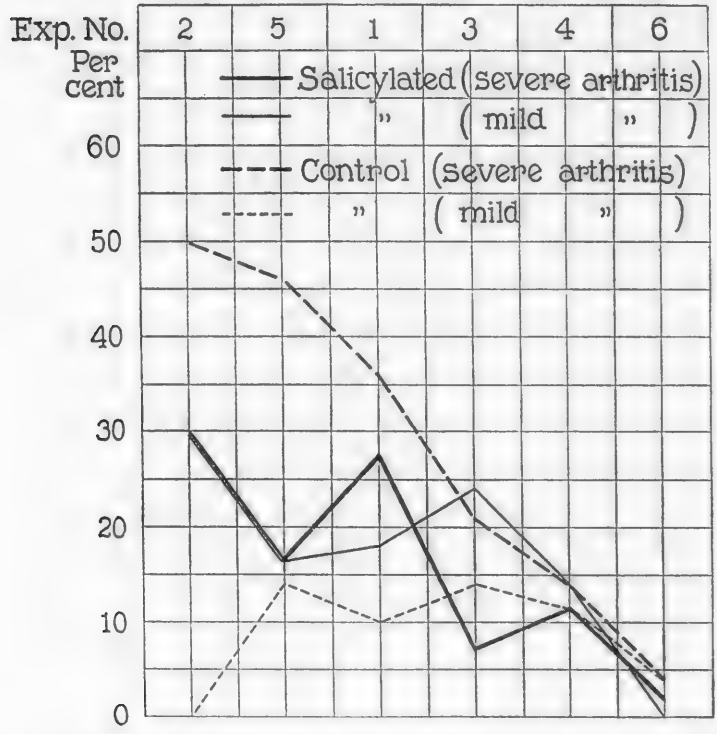


TEXT-FIG. 3. Percentage of inflamed joints among the rabbits in each experiment. Experiments arranged according to the degree of virulence of the streptococci.

to the fact that two of the S animals in this experiment died before sufficient time had elapsed to allow severe arthritis to develop; this point has been more fully discussed in the individual experiment. It is thus evident that the results indicated in Text-fig. 1 are not due to wide variations in individual experiments and cannot be attributed

to a different number of animals in the respective groups or to a wide variation in the virulence of the streptococci injected; the only remaining influence to be considered seriously is that of the salicylates.

The number of joints from which positive cultures were obtained was approximately the same in the two groups of rabbits, twelve for



TEXT-FIG. 4. Percentage of severe and mild arthritis among the animals in each experiment. Experiments arranged as in Text-fig. 3.

the S and thirteen for the C groups, divided among seven rabbits in each group. Thus, if we considered only the percentage of joints from which streptococci could be grown, no influence could be ascribed to the salicylates.

The foregoing evidence indicates that the salicylates modified favorably the inflammation induced in the joints of certain rabbits by nonhemolytic streptococci; this was less marked when organisms of higher virulence were employed, and could not be demonstrated when hemolytic streptococci were used. The negative results of Davis and of Fantus and his coworkers is, thereby, easily explained. It is necessary to balance properly the dose of both the drug and inoculum.

To what can this influence be ascribed? Two possibilities are suggested: (1) that the drug had a local antibacterial effect; (2) that it had a direct action on the tissues; *i.e.*, that it lowered the capacity of these tissues to react to the irritation of the streptococci. In the first instance, the action would be considered from a bacteriological, and in the second, from a physiological view-point. With the information at hand, it is difficult, if not impossible, to decide which of these two actions was the greater, or whether there may not have been a combination of the two. In each group of animals it was possible to cultivate streptococci from approximately 12 per cent of the inflamed joints. From the bacteriological examination of the joints of the controls it is evident that the normal resisting agencies of the animals caused the death of the invading microorganisms in the majority of instances. It seems probable that this bactericidal action occurs at different intervals following the invasion of the joints, for the most plausible explanation of the various degrees of severity of the local lesions is that the bacteria have acted for longer or shorter periods. Scott, Thoburn, and Hanzlik (14) have shown that salicyl occurs in demonstrable concentrations in the synovial fluid of salicylated animals. It is possible, therefore, that the addition of an antibacterial drug (even though weak as the salicyl must be in its existing concentration) to the normal bactericidal agencies of the body might lead to a more rapid destruction of the bacteria in the invaded joints, and consequently to a less severe inflammation in these joints. If, on the other hand, the number or virulence of the bacteria exceeded a certain level, an antibacterial action of the drug might fail of demonstration.

A second, or physiological action of the salicyl must be considered. Dohrn (15) has shown that benzoic and salicylic acids, given by mouth, have a strong antiphlogistic action on the conjunctivitis

induced by instillations of mustard oil into the conjunctival sacs of animals. He concludes, in general, that acids which possess antiseptic and antipyretic properties are also anti-irritants. The arthritis induced in rabbits by bacteria of relatively slight virulence might furnish suitable conditions for the demonstration of this anti-inflammatory action of salicyl. The inflammation might, on the other hand, be so severe that any local antiphlogistic action would fail of demonstration. One of us (11) has shown that inoculated animals, under the influence of salicylates, form fewer immune bodies than do unsalicylated controls. Such results are in line with those here reported. One might expect a drug which reduced the intensity of local lesions also to depress the capacity of the body to produce antibodies against the microorganisms inciting those lesions.

Those who regard non-hemolytic streptococci as the specific etiologic agents in rheumatic fever may consider that our experiments confirm their opinions, because an anti-arthritic action of salicyl was demonstrated in joints irritated by green streptococci. We think that one is not justified in reaching this conclusion for the following reasons: The arthritis was neither completely inhibited nor cured as is that of patients with rheumatic fever. Frequently in both the salicylated and control animals there was a purulent inflammation of the muscles contiguous to the involved joints; this and the severe types of arthritis found in many of our experimental animals are never found in patients with rheumatic fever. Until only milder types of arthritis responding completely to salicylates result from the experimental inoculation of animals, and the visceral lesions in these animals resemble more closely those of patients, one would prefer to leave this question open.

CONCLUSIONS.

1. Rabbits inoculated intravenously with non-hemolytic streptococci, while under the influence of full therapeutic doses of sodium salicylate, developed almost as many inflamed joints as the untreated controls similarly inoculated.

2. The salicylated rabbits, on the other hand, had a much higher proportion of mildly inflamed joints than did the controls.

3. This anti-inflammatory action was most evident in the animals inoculated with streptococci of the lowest virulence, and could not be demonstrated in animals inoculated with hemolytic streptococci.

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THE INFLUENCE OF CALCIUM CHLORIDE UPON EXPERIMENTAL BOTULISM.

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(Received for publication, November 6, 1922.)

For some time one of us has been interested in producing agglutinating serums for *Bacillus botulinus* in rabbits. After many failures, due mostly to the loss of animals from botulism during immunization, a successful method was finally found, the details and results of which will be reported separately.

One of the unsuccessful methods involved washed glucose broth cultures as immunizing antigens. In testing this method a certain rabbit was inoculated intravenously on February 25, 1921, with 1 cc. of a 1:10 dilution of a heavy suspension of *Bacillus botulinus* 78, Type B (see Hall (1) for origin and description), which had been freed from toxin by twelve repeated changes of 0.85 per cent NaCl by the centrifugal method. The dosage was rapidly increased in succeeding inoculations at intervals of about 6 days until in the last few injections, which terminated May 9 with the fourteenth dose, 2 cc. of undiluted suspension were given. No symptoms were observed at any time and the rabbit gained weight; but several trial bleedings during and following immunization failed to reveal agglutinins.

Having in mind recent reports of reactivation of detoxified spores of *Bacillus botulinus* in the animal body with their possibly important implications that this organism may invade the tissues and produce its toxin *in vivo*, we injected this animal subcutaneously on June 15 with 10 mg. of calcium chloride. Nothing developed, and on June 21, 20 mg. were given intravenously. The following day, June 22, muscular weakness was noted; this became progressively worse and simulated chronic botulism as seen in other rabbits. On July 7 the animal was nearly prostrate and was sacrificed. At autopsy

the left lung and brain were slightly hyperemic, as in botulism, and a marked induration was found at the point of injection of calcium chloride. It should be stated that the animal had severe auricular mange, but at autopsy no inflammatory extension to meninges or brain could be demonstrated. The results, while inconclusive, seemed to support the findings of Edmondson, Giltner, and Thom (2) but further and more carefully controlled experiments with guinea pigs have failed to do so.

HISTORICAL.

Nearly all the early work upon the infectivity of detoxified spores was done with *B. tetani*. Vaillard and Vincent (3) in 1891 showed that unheated cultures of *B. tetani* grown at 20–22°C. and that 37°C. cultures heated to 65°C. for 20 minutes are relatively non-toxic. Vincent (4) later found that guinea pigs injected with toxin-free spores of tetanus and kept at 40–42°C. develop tetanus, whereas those kept at ordinary temperatures do not. Tarozzi (5) and Canfora (6) found that heat-detoxified tetanus spores remained latent in the viscera of experimental animals as long as 3½ months after inoculation.

The reactivation of detoxified tetanus spores by lactic acid, trimethylamine, "*Microbacillus prodigiosus*," or even simple trauma had been shown by Vaillard and Vincent (3), and in 1911 Semple (7) added quinine to the list.

In 1914 Francis (8) showed that the "washing of tetanus spores by successive suspensions and centrifugalizations in large quantities of saline solution did not rid them of their toxin," and that tetanus spores detoxified by heating to 80°C. for 1 hour previous to injection may be reactivated by injection of quinine or staphylococci up to 1 month in guinea pigs and by staphylococci but not quinine up to 4 months in mice.

Koser and McClelland (9) in 1917 found that the spores of certain non-pathogenic aerobes disappear rapidly from the site of inoculation and from the internal organs, whereas those of *B. tetani* and certain other anaerobes persist both locally and in the viscera for some weeks or even months. Teale and Bach (10) in 1920 also concluded that washed spores of aerobes, including *B. anthracis*, do not usually remain latent in the animal body, many being rapidly phagocytosed and destroyed, while the rest vegetate and are then more slowly phagocytosed. They found that *B. welchii* and *B. tetani* behave like aerobes, except that a small, sometimes a large, proportion do not germinate but remain latent; there appeared to be no multiplication of the washed anaerobes in the tissues. Teale and Bach found several substances to influence infection and toxemia from washed spores in guinea pigs, including lactic acid, ferric chloride, calcium chloride, oleum phosphoratum (B.P.), given subcutaneously up to 4 days after the organisms, and gelatin, charcoal, or peptone, given intraperitoneally at the same time as the organisms.

In 1919 Bullock and Cramer (11) showed that surface cultures or washed broth cultures of *B. tetani*, *B. welchii*, and *vibrion septique* were non-infectious for mice and guinea pigs. The same was true of *B. œdematiens* (*B. novyi?*), but it was difficult to remove all the toxin by washing. They found that washed suspensions could be reactivated by the readdition of the culture fluid in an amount too small to have any effect of itself in an animal. Certain ionizable salts of calcium, such as the chloride, nitrate, or acetate, had the same effect when injected either with, previous to, or within a few days after the organisms. The insoluble salts of calcium, such as the carbonate, were impotent; the chlorides of sodium, potassium, ammonium, and magnesium were without effect; strontium chloride was found to act on *vibrion septique*, but only in larger dosage, and less uniformly than calcium chloride; citrates, given simultaneously with calcium ion, rendered the latter innocuous. Water extracts of earth were sometimes found to be potent, but apparently not always in proportion to the calcium content. Toxins, as of diphtheria, also induced "defense rupture," or "kataphylaxis," as this phenomenon was designated by them.

Bullock and Cramer found direct contact between the bacteria and calcium to be unnecessary, since positive results were obtained when injections were made at the same time into different sites, or at different times into the same or different sites. However, more prompt and uniform results were obtained by simultaneous introduction into the same site.

They showed also that toxin-free organisms suspended in calcium chloride solution, then centrifugalized out and injected, did not produce the disease, *i.e.* had not acquired virulence, although the rate of growth of the bacteria was accelerated. Neither did the administration of calcium chloride render animals more susceptible to injections of the bacterial toxin alone. From these results it was concluded that the calcium produced a local tissue change at the site of injection favoring invasion and toxin production, a conclusion supported by the observation that when the organisms and calcium chloride were injected separately bacterial multiplication took place and the disease localized where the calcium was introduced.

Bullock and Cramer interpreted the patchy distribution of tetanus and gaseous gangrene in the great war as an expression of the differing calcium content of the soil in various localities.

As regards *B. botulinus*, Van Ermengem (12) was able to destroy the toxicity of cultures by alkali, heating, or washing, but found that when the spores were protected from phagocytosis by injection in agar, or when they were injected with lactic acid, *B. prodigiosus*, or *Micrococcus tetragenus*, they failed to initiate the disease, as had then recently been shown for tetanus. Notwithstanding, Van Ermengem and others have occasionally recovered *B. botulinus* from the internal organs, but, owing largely to the belief, based upon Van Ermengem's work, that *B. botulinus* would not grow readily at the temperature of the animal body, the possibility of toxin-free spores, as in heated canned foods, causing

botulism has been generally disregarded. Recent speculations therefore have centered about the fact that the strains of *B. botulinus* recovered in the United States grow more rapidly at 37°C. than at 25°C. and with equally strong toxin production.

Suggestions of the possible pathogenicity of *B. botulinus* appeared in the work of Shippen (13), Armstrong, Story, and Scott (14), and Orr (15, 16), but the possibility of reactivation of toxin-free spores seemed proved by Edmondson, Giltner, and Thom (2) in their study of the action of $CaCl_2$. There was a remote possibility that some cases of botulism might be due to true infection.

EXPERIMENTAL.

The guinea pigs were, except when otherwise noted, young healthy animals, weighing from 250 to 350 gm. No record was kept of the exact weights.

While our own preliminary observation was upon a rabbit injected with Strain 78, Type B, this culture was not used again, because it fails to sporulate freely. Culture 80, Type B (see Hall (1) for origin and description), was therefore used throughout the following experiments.

The culture was planted in a meat mash broth in two 1 liter flasks (about 1,800 cc. in all) and incubated at 37°C. for 10 days. Two guinea pigs were given 0.001 cc. each of broth culture by mouth; one animal died, the other developed definite botulism, but finally recovered. The broth cultures were strained through gauze to remove the large particles, put in bottles, and centrifugalized at high speed for several hours. The organisms recovered were washed fourteen times in normal saline solution. We have found by much experience, as have Armstrong, Story, and Scott (14), and Edmondson, Giltner, and Thom (2), that a lesser number of changes usually fails to free the organisms from toxin. About one-fourth of the organisms were lost through breakage; the remainder were suspended in 100 cc. of 0.85 per cent NaCl, put in a large flask in a thin layer with considerable surface exposure to prevent multiplication, and kept in the ice chest. This suspension will be referred to as the original concentrated suspension; no accurate bacterial count was made, but the suspension was very concentrated, gray, and opaque. Spores were not so numerous as was hoped for, being present in the ratio, roughly, of one to eight vegetative forms. A dilution of 1:1,000 gave growth in brain medium on inoculation of 1 cc.

TABLE I.

Experiments with Washed Organisms.

Experiment No.	Guinea pig No.	Organisms. Amount.	Organisms. Route.	Calcium chloride. Length of time after administration of organisms.	Calcium chloride. Amount.	Calcium chloride. Route.	Result.
				days	*mg.*		
1	1	1 cc. of concentrated suspension.	Mouth.				Dead within 24 hrs.
	2	1 " " " "	Intraperitoneal.				" " 24 "
	3	1 " " 1:100 diluted "	Heart.				" on 2nd day.
	4	0.5 " " 1:100 " "	Intraperitoneal.				" " 2nd "
	5	0.2 " " 1:100 " "	"				" " 4th "
2	6	0.2 " " 1:100 " "	Heart.				Slight symptoms of botulism on 2nd day lasting a week. Recovered.
	7	0.2 " " 1:100 " "	"	9	30	Subcutaneous.	No symptoms of botulism.
3	8	1 " " 1:1,000 " "	"				
	9	1 " " 1:1,000 " "	"	4	20	"	" " " "
				6	30	"	" " " "
	10	1 " " 1:1,000 " "	"	At once.	20	"	" " " "
4	11	1 " " 1:1,000 " "	Intraperitoneal.				" " " "
	12	1 " " 1:1,000 " "	"	At once.	55	Intraperitoneal.	" " " "
	13	1 " " 1:1,000 " "	Pleural cavity.	7	20	Subcutaneous.	" " " "

Experiments with Washed Organisms.

The toxicity of this suspension was tested by different routes and in various doses, as shown in Experiment 1, Table I.

The data indicate that the suspension, although washed fourteen times, was still highly toxic. In order to determine whether free toxin was present, some of the suspension was filtered, together with *Bacillus prodigiosus* as a filter control, through a small Mandler candle. No growth could be obtained from the filtrate, either in brain medium or on agar slants, and a guinea pig showed no symptoms after receiving 2 cc. by mouth. It was therefore considered that whatever toxin was present was closely fixed upon the bacteria.

These first few animals served to establish the maximum non-symptomatic dose of suspension as about 0.2 cc. of 1:100 or 1 cc. of 1:1,000 dilution. Certain guinea pigs that had been safely injected by cardiac puncture with these doses without developing botulism were given, after varying periods as indicated, differing doses of calcium chloride solution, made up in boiled distilled water to avoid possible carbonate precipitation. As may be seen in Experiments 2 and 3 (Table I) when a sublethal dosage of *Bacillus botulinus* was used the subsequent injection of calcium chloride had no influence on the result. It is admitted that the time interval between injection of organisms and injection of calcium salt was in some cases greater than was reported to give pronounced results. Furthermore, our experiments differed from those of Bullock and Cramer and Edmondson, Giltner, and Thom in that they gave the bacteria subcutaneously. Teale and Bach, however, gave both intraperitoneal and intravenous inoculations, but did not study *Bacillus botulinus*.

Since there were no obvious advantages in the method of cardiac inoculation, and since it was attended by some immediate mortality, it was discarded for the simpler intraperitoneal route. However, the single experiment upon washed organisms by this method was equally discouraging, as seen in Experiment 4 (Table I).

Experiments with Organisms Detoxified by Alcohol.

Following Bronfenbrenner and Schlesinger's (17) report that ethyl alcohol destroys *botulinus* toxin, we decided to try this method

of detoxification. 10 cc. of the original concentrated suspension were mixed with 10 cc. of 50 per cent ethyl alcohol and allowed to stand 10 minutes at room temperature, then diluted to 100 cc. with normal saline solution to stop the action of the alcohol, centrifugalized, washed once, and made up to 20 cc. with normal saline solution. Thus, in addition to treatment with alcohol, these organisms received two extra washings, making sixteen in all. The concentrated alcohol-treated suspension so obtained was one-half the concentration of the original, less the organisms lost in washing. In view of the possibility of killing vegetative forms with the alcohol, comparable dilutions were made of the original and the alcohol-treated suspensions and similar amounts were inoculated into deep agar, but the growth from the alcohol-treated organisms was approximately the same as that from the original.

In Experiments 1 and 2 (Table I) it was found that 0.2 cc. of 1:100 diluted suspension was pathogenic in some cases; in Experiment 7 (Table II) 2 cc. of 1:50 dilution (equivalent to 2 cc. of 1:100 of the original) were on the borderland of infectivity. It seems, therefore, that the alcohol reduced the toxicity of the suspension to one-tenth its original value without injuring the organisms.

As will be seen from Table II, little if any effect was obtained from using calcium chloride, though in Experiment 6 the animal receiving calcium died promptly, as if by "defense rupture." However, in Experiment 7 a slightly greater dilution of the suspension gave an inconclusive result; one control developed no symptoms, and one died, while the results with the animals receiving calcium chloride were fair duplicates of those with the controls. Experiments 8 and 9 indicate that a dosage only slightly below that occasionally producing symptoms is certainly not influenced by calcium chloride.

Experiments with Heated Organisms.

As an alternative method of reducing the toxicity of the washed organisms from time to time as needed, samples of the original concentrated suspension were placed in test-tubes and heated in a water bath at 80°C. for 15 minutes. As has been repeatedly shown, *botulinus* toxin is very susceptible to heat; Orr (15) found that 2 minutes at 80°C. was usually sufficient to destroy it. Heating for 15 minutes

TABLE II.

Experiments with Organisms Detoxified by Alcohol.

Experiment No.	Guinea pig No.	Organisms. Amount.	Organisms. Route.	Calcium chloride. Length of time after administration of organisms.	Calcium chloride. Amount.	Calcium chloride. Route.	Result.
					mg.		
5	14	0.5 cc. of concentrated suspension.	Intraperitoneal.				Dead on 2nd day.
	15	0.4 " " " "	"				" within 24 hrs.
	16	0.2 " " " "	"				" " 24 "
	17	1 " " 1:10 diluted "	"				Died on 2nd day.
	18	1 " " 1:10 " "	"				Dead on 3rd "
6	19	0.5 " " 1:10 " "	"				No symptoms.
	20	0.5 " " 1:10 " "	"				Slight muscle flabbiness developed in 2 days; lasted 10 days. Recovered.
	21	0.5 " " 1:10 " "	"	At once.	27	Intraperitoneal.	Dead on 3rd day.
7	22	2 " " 1:50 " "	"				No symptoms.
	23	2 " " 1:50 " "	"				Weakness in 4 days. Sacrificed on 15th day.
	24	2 " " 1:50 " "	"	At once.	55	Intraperitoneal.	No symptoms.
	25	2 " " 1:50 " "	"	At once.	27	Subcutaneous.	" " for 2 wks. Died on 15th day.*

8	26	1	cc. of	1:50	diluted	suspension.	Intraperitoneal.				No symptoms.
	27	1	"	1:50	"	"	"	At once.	30	Subcutaneous.	" "
9	28	0.5	"	1:50	"	"	"				" "
	29	0.5	"	1:50	"	"	"				" "
	30	0.5	"	1:50	"	"	"	At once.	20	Subcutaneous.	" "
	31	0.5	"	1:50	"	"	"	At once.	20	"	" "

* Animal did not die from botulism; cause uncertain.

TABLE III.

Experiments with Heated Organisms (Concentrated Suspensions).

Experiment No.	Guinea pig No.	Organisms.		Calcium chloride.			Result.
		Amount.	Route.	Length of time after administration of organisms.	Amount.	Route.	
		cc.			*mg.*		
10	32	1	Intraperitoneal.				Dead on 5th day. *B. botulinus* isolated from heart's blood post mortem.
11	33	0.5	"				Dead on 6th day.
	34	0.5	"				Slight muscle weakness developed in 1 wk.; lasted about 1 wk. Recovered.
	35	0.5	"				Dead on 7th day. *B. botulinus* isolated from heart's blood post mortem.
	36	0.5	"	At once.	55	Intraperitoneal.	Dead on 4th day. Young, small animal.
	37	0.5	"	" "	41	"	" within 3 days. Young, poorly nourished animal.
	38	0.5	"	" "	27	Subcutaneous.	Dead on 8th day.
	39	0.5	"	" "	27	Intraperitoneal.	No symptoms.
	40	0.5	"	" "	55	"	Dead on 10th day.
	7	0.5	"	" "	20	Subcutaneous.	No symptoms. Had previously received a single dose of washed organisms in heart (see Experiment 2, Table I).
	41	0.5	"	" "	55	Intraperitoneal.	No symptoms.

12	42	0.4	Intraperitoneal.				No symptoms.
	43	0.3	"				" "
	44	0.3	"	At once.	55	Intraperitoneal.	" "
13	45	1	Mouth.				" " of botulism; developed respiratory symptoms and died with streptococcic septicemia on 23rd day.
	46	3	"				No symptoms.
	47	3.5	"				" "
	48	2	"	At once.	55	Intraperitoneal.	" " Killed through error on 18th day.

undoubtedly also destroys many, if not all, of the vegetative forms, as well as the toxin.

The data in Table III indicate that the heated spores of *Bacillus botulinus* 80 were able to produce the disease when given intraperitoneally, but that the doses given by mouth had no effect.

In the experiments recorded in Table III no effect could be attributed to the calcium chloride, for while Guinea Pigs 36 and 37 died considerably sooner than the controls, they were both underdeveloped; furthermore, the negative results with several other animals tend to offset these two.

The infective dose of the heated organisms was about twenty times that of the alcohol-treated, owing probably to the elimination of vegetative forms in the heated suspension, but possibly in part to destruction of final traces of toxin.

Bacillus botulinus was isolated from the heart's blood procured at autopsy in two of the cases in Table III. This is not a common finding, but has been reported in the literature (13, 15). We attach no great significance to the occurrence of *Bacillus botulinus* in the blood in our cases (two guinea pigs and one rabbit), since the animals in each instance had been dead several hours before examination and there is no satisfactory evidence that the organisms were circulating in the blood stream at the time of death. Two guinea pigs were autopsied immediately following death from botulism, and several cubic centimeters of blood aspirated from the heart. Both samples were negative for *Bacillus botulinus*. One of us in other experiments has made numerous heart's blood cultures from animals dead or dying from botulism with uniformly negative results.

DISCUSSION.

The purpose of these experiments was to study the action of calcium chloride in initiating "defense rupture" to *Bacillus botulinus*. No such action has been demonstrated, although the doses given were sufficiently large to cause induration and in some cases sloughs by the subcutaneous route.

As previously noted, the present work differs from that of Edmondson, Giltner, and Thom (2) in the details of injection of organisms and calcium chloride, but it scarcely seems probable that these

differences can account for the discrepancy in results. It is possible, however, that the difference can be explained upon the basis of strain variability. The forenamed authors were careful to state that one of the strains with which they worked showed rather inconstant results.

We have confirmed the statement of Orr (15, 16) and of Edmondson Giltner, and Thom (2) that toxin-free spores of *Bacillus botulinus* are able to produce botulism on injection. We were unable to demonstrate this by feeding the spores; it seems, therefore, that the possibility of human disease contracted from eating freshly cooked food contaminated with *Bacillus botulinus* would be slight, as suggested by Burke, Elder, and Pischel (18).

It has been found that *botulinus* organisms washed as many as fourteen times and free from diffusible toxin are still highly toxic to guinea pigs. Treatment of the organisms with alcohol reduces the toxicity out of all proportion to demonstrable injury to bacteria.

The course of the disease in experimental animals has been almost unpredictable. Sometimes a guinea pig that shows marked symptoms after 4 to 5 days will go on to recovery; in other instances an animal progressing favorably will take a sudden turn for the worse and become prostrated within a short while. Occasionally an animal will show no symptoms for 10 days or 2 weeks, then be taken severely ill and die within 24 hours. These sudden unforseen fatalities have been interpreted by competent observers as due to thrombosis or embolism.

In diagnosis reliance has been placed largely on muscular weakness, noticed especially in flaccid, dragging abdomen, hollow flanks, and difficulty in walking. Later the animals often show dyspneic respiration and dribbling salivation. At autopsy the lesions are not altogether distinctive; perhaps the most constant finding is hyperemia of the lungs and pericardium, and sometimes hemorrhages in these localities. The liver may be engorged, the spleen, kidneys, and brain hyperemic, and the mesentery and intestines occasionally injected.

SUMMARY.

1. Calcium chloride given subcutaneously, intraperitoneally, or intravenously has been found to have no effect upon the production

of botulism following the injection of *Bacillus botulinus* (Strain 80B) into the peritoneal cavity of guinea pigs.

2. Treatment of *Bacillus botulinus* with alcohol has been found markedly to decrease its toxicity for guinea pigs. This is in conformity with the work of Bronfenbrenner and Schlesinger.

3. Toxin-free spores of *Bacillus botulinus* have been found pathogenic for guinea pigs.

4. No prejudice as to possible results in rabbits should be based upon the above conclusions.[1]

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[1] Since this paper was written Coleman and Meyer (Coleman, G. E., and Meyer, K. F., *J. Infect. Dis.*, 1922, xxxi, 622) have shown also that "massive doses of toxin-free spores of *B. botulinus* are pathogenic when introduced into the animal body." We are not ready to agree, however, with their last conclusion, namely that "toxin-free spores of *B. botulinus* germinate, and the vegetative forms arising from this germination multiply and liberate toxin in the animal body," which seems based upon insufficient evidence.

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Avenue A and 66th Street, New York, N. Y.